Development of a Tissue Engineered Pancreatic Substitute Based on Genetically Engineered Cells

A Thesis
Presented to
The Academic Faculty

By

Shing-Yi Cheng

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in
Chemical & Biomolecular Engineering

Georgia Institute of Technology
August 2005

Development of a Tissue Engineered Pancreatic Substitute Based on Genetically Engineered Cells

Approved by:

Dr. Athanassios Sambanis, Advisor
School of Chemical & Biomolecular Engineering
Georgia Institute of Technology

Dr. Timothy M. Wick
School of Chemical & Biomolecular Engineering
Georgia Institute of Technology

Dr. Peter M. Thulé
Division of Endocrinology and Metabolism
Emory University and Atlanta VA Medical Center

Dr. L. Andrew Lyon
School of Chemistry & Biochemistry
Georgia Institute of Technology

Dr. Elliot L. Chaikof
School of Chemical & Biomolecular Engineering
Georgia Institute of Technology

Date Approved: June 22, 2005

To Mom and Dad

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere appreciation and gratitude to my advisor, Dr. Athanassios Sambanis, for his patience and kindness in helping me grow professionally during my PhD studies. I will always remember how he smiled at me and encouraged me to work hard and strive for excellence. He has been a wonderful advisor and I am very fortunate to have the opportunity to work with him. I would also like to thank Dr. Ioannis Constantinidis and my committee members, Dr. Timothy Wick, Dr. Peter Thule, Dr. Andrew Lyon, and Dr. Elliot Chaikof, for their input and assistance in this work.

Special thanks go to my former lab members, Dr. Cherie Stabler, Dr. Tony Tang, and Dr. Joe Berglund, for their wonderful friendship and guidance since the beginning of my studies. I thank the members of Dr. Sambanis' group, Jeff Gross, Neil Mukherjee, Heather Virginia, Angela Chu, and many others, for their friendship and help in the lab. Additionally, I would like to thank the faculty, staff, and friends in the School of Chemical & Biomolecular Engineering and GTEC for enriching my life at Georgia Tech.

I would like to acknowledge my family, whose love and support have helped me stay strong in pursuing my dreams. Even though my grandmother, Bao-Lien Tsao, passed away four years ago, memories of her love and support are always deep inside me. I am grateful to my Dad, Dr. Ying-Ming Cheng, for his words of wisdom and for being the role model in my life. Finally, I would like to thank my Mom, Chiu-Ching Lin. If

I have any achievement in my life, it would be due to her unconditional love and support.

This work was supported by the ERC Program of the National Science Foundation under Award Number EEC-9731643, by NIH grants DK56890, and by the Juvenile Diabetes Foundation. Partial stipend support in year 2004 was provided by GAANN fellowships.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SUMMARY

Genetically engineered cells have the potential to solve the cell availability problem, which exists with islets of Langerhans in developing a pancreatic tissue substitute for the treatment of insulin-dependent diabetes (IDD). These cells can be β cells genetically engineered so that they can be grown in culture, such as the βTC3 and βTC tet mouse insulinomas developed by Efrat et al; or, they can be non-β cells genetically engineered to secrete insulin constitutively or under transcriptional regulation. As insulin secretion needs to be tightly regulated by glucose and other physiologic stimuli in order to achieve normoglycemia in higher animals and, eventually, humans, the aim of this work was to thoroughly characterize and improve the secretion dynamics of pancreatic substitutes based on genetically engineered cells.

One issue involved with the continuous β-cell line is the remodeling of the cells inside an encapsulated cell system, which may affect the insulin secretion dynamics exhibited by the construct. To evaluate the effect of remodeling on the secretion properties of the construct, we used a single-pass perfusion system to characterize the insulin secretion dynamics of different alginate beads in response to step-ups and downs in glucose concentration. Results indicated that the secretion dynamics of beads indeed changed after long-term culture. On the other hand, data with growth-regulated cell line, βTC tet cells, showed that the secretion profile of beads can be retained if the cell growth was suppressed.

A major concern associated with genetically engineered cells of non-β origin is that they generally exhibit sub-optimal insulin secretion characteristics relative to normal

pancreatic islets. They are either secreting insulin constitutively or exhibiting slow responses to changes in the concentration of secretagogues. To generate pancreatic substitutes with appropriate secretion dynamics based on such sub-optimal cells, a novel idea is proposed, which consists of sequestering these cells from the surroundings by a glucose-responsive material serving as a controlled barrier for insulin release. The idea of improving the secretion dynamics by combining cells with materials, rather than relying on molecular tools, bears similarities to insulin pumps. For an insulin pump, fluctuations in glucose concentration are picked up by the glucose sensor, whose output signal is sent to the controller that regulates the pump releasing insulin from a reservoir. In our proposed tissue engineered construct, the glucose-responsive material has the role of both the glucose sensor and controller, and the cells inside the construct serve as a continuously refillable insulin reservoir. Since the cells used in the construct are of potentially autologous origin, they relax the immune acceptance problems intrinsic to the use of xeno- or allogeneic cells.

The proposed first prototype of hybrid construct consisted of insulin-secreting cells sequestered from the surroundings by silicone, a 3 kDa MWCO membrane permeable to glucose and oxygen but not to insulin, and a material barrier sandwiched between two insulin-permeable membranes. Cells used in these studies were HepG2 human hepatomas and C2C12 murine myoblasts, both genetically engineered to secrete insulin constitutively. Cells were encapsulated in approximately 700 μm in diameter alginate beads prior to incorporation in the device. To study the feasibility of developing a cell-material hybrid pancreatic construct, a glucose-responsive material based on concanavalin A was used.

The cell-material hybrid construct released insulin at different rates that depended on the glucose concentration in the surrounding medium. Following a glucose step up, the con A-based material experienced a gel to sol transformation, where the sol-phase material was more permeable to insulin, thereby allowing a higher insulin release rate. Removal of glucose caused the material to change back to gel reducing the rate of insulin transport and thus the release of insulin from the device. In experiments with a control construct, in which the material barrier consisted of an alginate sheet, the rate of insulin release from the device did not respond to glucose concentration changes. These initial secretion experiments indicate feasibility of engineering a properly secreting pancreatic substitute based on sub-optimally secreting cells.

As the con A-based material used in the proof-of concept experiments exhibited glucose sensitivity far beyond the physiologic range, the last part of the thesis attempt to addresses this issue by using PEGylated con A molecules. Material formed with PEGylated con A has improved glucose sensitivity, and the hybrid construct containing modified con A-based material also released insulin in response to step changes of glucose concentration closer to physiological range. Although there is still much work to be done with the development of cell-material hybrid construct, we have successfully presented a new set of methodologies for engineering a pancreatic substitute potentially based on autologous non-β-cells.

CHAPTER 1

INTRODUCTION

Diabetes mellitus (DM) is a serious health problem affecting more than 18.2 million people and costing over 132 billion dollars a year in the United States. The cause of the disease has been associated with both genetics and environmental factors such as obesity and lack of exercise. Unfortunately, as shown by the increased rates of obesity and the trend toward more sedentary lifestyles, its incidence will continue to grow. Patients who suffer from DM are characterized by abnormally high levels of blood glucose because the body does not produce or properly use insulin. About 31 % [1] of DM patients are taking insulin to maintain their healthy state, and these populations are so called the insulin-dependent diabetics (IDD). However, a study done by Diabetes Control and Complications Trial Research group has shown that taking one or two shots of insulin a day is not enough to prevent long-term complications associated with hyperglycemia, including retinopathy, nephropathy, neuropathy, and cardiac and peripheral vascular disease [2]. A proper regimen suggested in that study included three or more insulin injections per day or continuous subcutaneous insulin infusion, in addition to frequent blood glucose testing to achieve normoglycemia. Clearly, these treatments are cumbersome to the patient, and they also have the adverse effect of increasing the probability of severe hypoglycemic episodes two to three fold relative to traditional insulin therapy.

Insulin plays a major role in regulating blood glucose level, as blood-glucose level fluctuates depending on the caloric intake and expenditure, too much or too little insulin

in the body could lead to hypo- or hyperglycemia, respectively. To prevent both conditions, an insulin-delivery system should be well designed to mimic how β cells release insulin, in which cells not only secrete insulin at a low rate during basal blood glucose but also respond quickly to the increase and the decrease of blood-glucose level. Based on this concept, closed-loop insulin delivery systems, consisting of a glucose sensor, a controlled pump, and an insulin reservoir, were proposed since the 80s'. However, some technical problems including, among others, the longevity and the stability of sensor, have not yet been overcome successfully.

Cell-based therapy has long been considered as the most promising approach in treating IDD. One of the success trials is the islet transplantation based on Edmonton protocol. About 80% of patients who received islet transplantation have become insulin independent up to a year [3]. However, in this treatment, patients are required to take lifetime immunosuppressive drugs, which could cause severe side effects after long-term usage. It is still up to debate whether the benefit of islet transplantations could outweigh its risk, however, based on the number of donors available per year, it is estimated that only 0.5% of patients are able to receive the treatment [4]. Thus, an alternative cell source needs to be pursued.

Various cell sources have been proposed that could be categorized into three major areas: xenogeneic islets, insulin secreting cells derived from stem cells, and genetically engineered cells. Each of the cell sources has limitations that need to be overcome. In this thesis, the focus is on the insulin secretion properties of genetically engineered cells. Two types of cells were studied: continuous β-cell lines and the cells of non-β origin.

Most of the currently available continuous β-cell lines have been derived from

transgenic animals, such as the βTC family developed by Efrat et al [5]. These cells are easy to culture and propagate, and have high insulin secretion rate with glucose responsiveness. Because developing a human continuous β-cell line has been proven to be quite challenging, this approach would eventually be adapted as xenogeneic cell transplantation. To prevent immune rejection, cells will need to be encapsulated in a semi-permeable membrane that allows low molecular-weight nutrients and insulin to pass through but not the penetration of the host cells or large molecular-weight antibodies. One of the promising approaches is to encapsulate these cells in alginate/ poly-L-lysine/ alginate (APA) microbeads. Nonetheless, as the cells would continue to proliferate after encapsulation, extensive cell-mediated remodeling could occur, including the development of nutrient gradients within the beads, degradation of the supporting matrix, and accumulation of cell death byproducts. All of these could eventually affect cellular phenotype or implant performance. Since insulin secretion dynamics are critical at restoring normoglycemia *in vivo*, especially in higher animal models, in Chapter 3 of this thesis, we built a perfusion system to address the effects of long-term culture on the secretion profile of alginate-encapsulated βTC cells.

Another genetically engineered cell type studied in this thesis is cells of non-β origin. Various cell types can be used, such as hepatocytes and myoblasts, hence less concern of cell availability. Cells can be taken as biopsy from a patient and genetically engineered *ex vivo* to become insulin-secreting cells. Thus, these autologous cells relax the immune-acceptance problems intrinsic to the use of xeno- or allogeneic cells. Nonetheless, as β cells are a highly differentiated cell type with a sophisticated phenotype in regulating insulin release according to metabolic cues, researchers have faced

tremendous challenges in using molecular tools, such as gene manipulation, to engineer these cells to have a proper insulin secretion dynamics: cells are either secreting insulin continuously or exhibiting sluggish kinetics in response to stimuli [6, 7].

The other parts of the thesis proposed a novel approach of incorporating a glucose-responsive material to improve the secretion profile of genetically engineered cells of non-β origin. Glucose-responsive material is a type of smart hydrogel that has different insulin-permeability under different glucose concentrations: at low glucose concentrations, the material is in a gel state that exhibits low insulin permeability, whereas at high glucose levels the material becomes a sol that exhibits a higher permeability to insulin. The time constant of the gel to sol and of sol to gel transformations is shorter than the (infinite) time constant of constitutively secreting cells or the (long) time constant of transcriptionally regulated cells. Through proper engineering, we can then develop a cell-material hybrid construct in which the kinetics of material transformation determine the kinetics of insulin release from the entire device, and hence improve the secretion characteristics exhibited by the cells alone.

As glucose-responsive materials have already been developed for the use of glucose sensor and insulin delivery, the proof-of-concept experiment began with identifying a type of glucose-responsive material suitable for associating with insulin-secreting cells. Selection criteria were listed, such as glucose specificity, kinetics of phase transformation, function under physiologic conditions, and capability of regulating insulin secreted by the cells, in other words: insulin does not need to be modified. Table 1.1 summarizes the results of the comparisons among three major types of glucose-responsive materials. Consequently, concanavalin A (con A)-based material, although not ideal, seems to be the

most suitable material for developing a cell-material hybrid construct. In CHAPTER 4, a diffusion chamber was built to test the glucose-responsiveness of con A-based material, and a preliminary experiment was done with encapsulated βTC3 cells as insulin source to test our hypothesis: glucose-responsive material can be used to control insulin released by the cells. A mathematical model was also presented to further demonstrate our idea.

Table 1.1 Comparisons of three major types of glucose-responsive materials

	Glucose specificity	Kinetics	Fuctions under physiologic conditions	Insulin modification
Con A-polysaccharides [34-36]	Good	Needs improvement	Acceptable	No
Glucose oxidase [15,16, 20, 21]	Good	Slow	Needs improvement	Yes
Phenyl-boronic Acid (PBA) [26-29]	Poor	Slow	Poor	Yes

The research done in CHAPTER 4 leads to the development of the first prototype of cell-material hybrid construct. Cells of non-β origin, human HepG2 hepatomas and murine C2C12 myoblasts, were genetically engineered to become constitutively insulin-secreting cells. By incorporating these cells in a hybrid construct containing con A-based material, we obtained a device that released insulin in response to glucose square-wave changes. The details are describes in CHAPTER 5. Although we were able to prove our concept by using con A-based material as a controlled barrier for the insulin released by the cells, problems exist with the material that need to be overcome

prior to any *in vivo* experiments, which include glucose sensitivity, stability, and biocompatibility.

In terms of glucose sensitivity, as the glucose responsiveness of con A-based material arises from the competitive binding reaction between free glucose and pendent glucose on a polymer chain toward con A binding sites, it is possible to improve the sensitivity of the material by using different types of polysaccharides or modified con A molecules that have different glucose binding affinity. For the latter, Park et al [8] reported that one can increase the binding affinity of con A towards free glucose up to 5 fold through PEGylation of con A. PEGylation is a well established tool in improving the biocompatibility and the stability of proteins, as well as a substrate for tethering protein to the surface of biomaterials. Inspired by these positive results from PEGylation, studies in CHAPTER 6 aimed at improving the glucose responsiveness of con A-based material through the use of PEGylated con A molecules. Other approaches taken to improve the properties of con A-based material are presented in APPENDIX A.1 and A.2.

General information on IDD treatment, including current therapies, development of glucose-responsive materials, and cell-based approaches are reviewed in CHAPTER 2, the BACKGROUND section. Conclusions and future work are described in CHAPTER 7.

CHAPTER 2

BACKGROUND

2.1 Diabetes Mellitus

Diabetes mellitus (DM) is a metabolic disorder associated with insufficient insulin secretion in response to glucose and tissue insensitivity to insulin action. It is recognized as one of the most expensive illnesses in the heath-care system, with 18 million people affected and 132 billion dollars spent in the United States of America [9]. Two main types of diabetes are Type 1 diabetes and Type 2 diabetes. Type 1 diabetes comprises 5-10% of DM and often develops in patients of young age and thus is commonly called "juvenile diabetes". It is an autoimmune disorder in which activated T-cells infiltrate the islets of Langerhans and destroy the insulin-producing β cells. The consequence of β-cells destruction causes the Type 1 diabetic patients to be completely insulin deficient, and as a result, patients are required to take daily insulin injections to maintain survival. Type 2 diabetes is the most common type of diabetes, which affects 90-95% of diagnosed diabetes. Its exact cause is still uncertain but is associated with a combination of genetic and nongenetic factors, the latter including increasing age, high caloric intake, and overweight. In contrast to type 1 diabetes, patients with type 2 diabetes usually produce some insulin, but their body cells cannot use it efficiently because the cells are resistant to insulin. By losing weight, controlling diet, exercising, and taking oral medications, it is possible for a patient to overcome the disease. Nonetheless, some type 2 diabetes patients gradually develop into insulin dependency, which is associated with impaired β-cell function, and hence require daily insulin

injections to stay alive. Type 1 diabetics together with type 2 diabetics who are required to take insulin injections are called insulin-dependent diabetics (IDD), and they account for about 31 % of DM cases [2].

In a healthy individual, β cells secrete insulin in response to blood glucose levels. Between meals or through out the night when the blood glucose is at the basal level, insulin is secreted continuously at a low rate. After a meal, cells adjust their insulin secretion rate according to the higher blood glucose concentration, with a threshold value at 4 mM, a half-maximal response at 8 mM, and a maximal rate at 15 mM. The kinetics of insulin secretion is very rapid both upon stimulation and removal of glucose. As the blood glucose level fluctuates a lot depending on a person's caloric intake and expenditure, patients who take only insulin injections are not able to maintain euglycemia, and eventually suffer from long-term complications such as heart, kidney, and nervous system diseases, stroke, and blindness.

Data from the Diabetes Control and Complications Trial (DCCT) [1] indicated that intensive treatment of type 1 diabetes with control of blood glucose significantly delays the onset of complications in diabetic patients such as retinopathy, nephropathy, and neuropathy by 50 to 75% when compared with conventional treatment. In contrast to the conventional treatment, which involves no more than two insulin injections per day, intensive therapy in the DCCT included multiple injections of insulin three or more times per day or continuous subcutaneous insulin infusion (CSII) by external pump, in addition to frequent blood glucose self-monitoring. However, the improvements of intensive therapy were associated with threefold increase in the risk of hypoglycemia and incidence of becoming overweight [10]. Hence, various alternatives are being developed to seek a

better control of insulin delivery that would eventually mimic the physiologic response of β cells. Following is a short overview of approaches and limitations of available treatments such as multiple insulin injections and insulin pumps, and the potential of cell-based therapies such as islet transplantations or the implantation of other insulin-secreting cells.

2.2 Multiple daily injections and insulin pump

In order to mimic the insulin secretion characteristics of β cells, in which a basal insulin secretion is maintained between meals and during bedtime, and bolus insulin release occurs after a meal, patients need a combination of both long-acting and short-acting insulin. The long-acting insulin, such as NPH, is used to provide the basal insulin needs. The short-acting insulin, such as Lispro, is taken before the meals for the postprandial insulin [11]. With this regimen, a patient needs more than three times of insulin injections per day, frequent glucose monitoring that involves finger poking, and the awareness of diet composition to determine the dosage required to prevent the event of hypo-or hyperglycemia. Undoubtedly, the amount of time and money involved with daily insulin-injections could deprive one's quality of life.

Another type of insulin delivery is the continuous subcutaneous insulin infusion (CSII) by external pumps. These are the battery-operated devices that function by delivering regular insulin through a needle inserted in the skin. Depending on a patient's need, the pump is programmed to deliver insulin at a basal rate during the fasting state, and before meals it can be programmed to infuse bolus doses of insulin to cover the carbohydrate content in the food. Although CSII seems to provide patients

with a better lifestyle, it poses the risks of skin irritation, infection at the infusion site, and pump failure, and has also the disadvantages of the high cost and limitation of physical activity because pumps can be jostled loose from the body [12].

Under those circumstances, an implantable insulin pump (IIP) that could continuously deliver insulin in response to glucose is considered as another possible replacement of insulin injections. An IIP should consist of three components: a pumping system that stores and releases insulin; a glucose monitoring system; and an algorithm that links the two systems together to ensure the right amount of insulin being delivered. Figure 2.1 shows the basic design of a typical implantable insulin pump. The pump is surgically implanted in a subcutaneous pocket in the lower abdomen under local anesthesia. A catheter linked to the pump is advanced through the rectus muscle into the peritoneal cavity. The peritoneal route is preferred over the subcutaneous route because insulin is absorbed by the portal system, which is more similar to pancreatic insulin secretion. Because insulin delivered would be adjusted based on the signal sent by the glucose sensor, the success of insulin-delivery from such system relies on the development of a continuous glucose-monitoring system. However, most of the glucose sensors developed up to date face the problems of short life-time, low stability, and problems of accuracy, thus limiting the development of implantable insulin pump. Besides the issues of developing a functional glucose-sensor, there exist other concerns with IIP, such as pumps require significant maintenance in that they need to be refilled and replaced; possible complications also involve catheter blockage, pump-pocket events, and pump mechanical failures. These, as well as the high cost of the device, limit the widespread applications of implantable systems [13, 14].

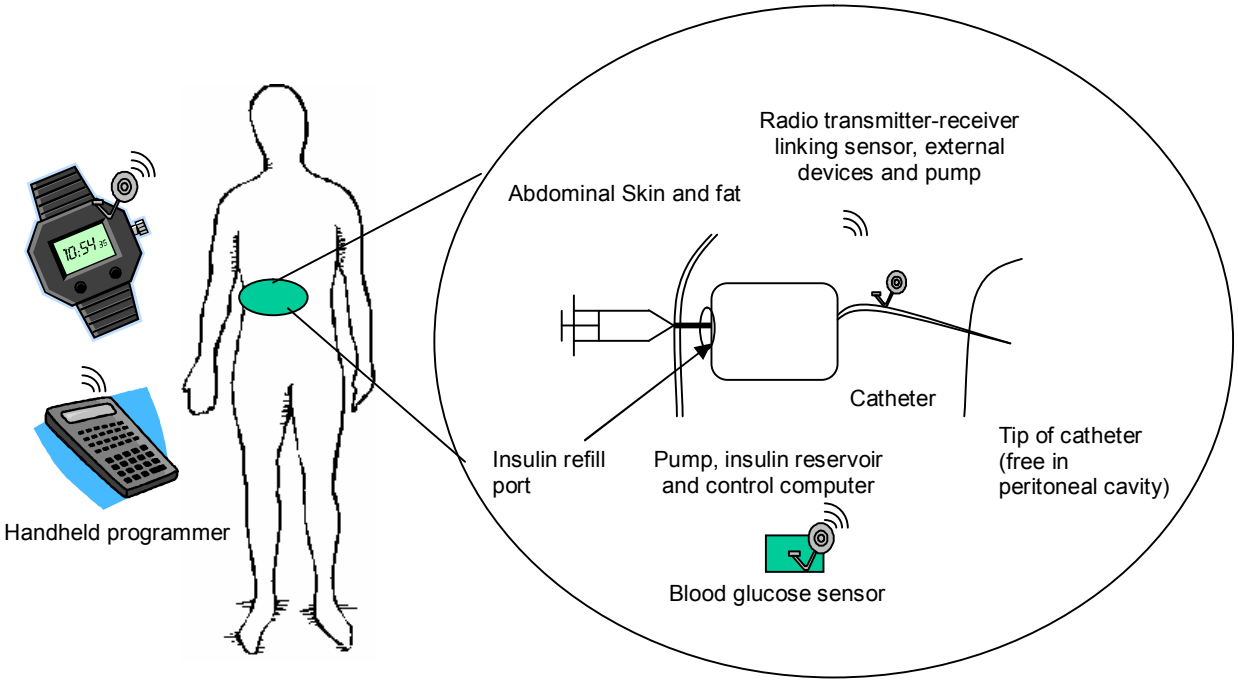


Figure 2.1 Schematic of implantable insulin pump.

2.3 Glucose-responsive material

As mentioned above, the major hurdles for an insulin pump to function properly are the performance of the glucose sensor and issues with the pumping system. To simplify a closed-loop insulin delivery without these two major components, another closed-loop system composed mainly of a glucose-responsive material has been proposed. In such a system, so called the self-regulated insulin-delivery, the glucose-responsive material has both roles of a glucose-sensor and a controlled barrier. Based on this concept, various novel designs, including bulk hydrogels, matrix systems, and membranes, were proposed for glucose-regulated insulin delivery. The performance of such systems differs based on the types of glucose-sensing mechanisms intrinsic to the materials used. The following paragraphs provide brief descriptions of three major types of glucose-responsive materials based on: glucose oxidase (GluOx), phenyl boronic acid (PBA), and concanavalin A (con A).

2.3.1 Material based on enzymatic reactions of glucose-oxidase (GluOx)

GluOx is a glucose-specific enzyme that catalyzes the following chemical reaction:

$$\textbf{Glucose} + O_2 + H_2O \rightarrow \textbf{Gluconic acid} + H_2O_2$$

Based on this enzymatic reaction, when a system containing GluOx is exposed to a higher glucose-concentration, the pH of the surrounding solution decreases due to the production of gluconic acid. Owing to this unique relation between pH and glucose concentration caused by the GluOx, different types of GluOx-containing insulin delivery systems have been proposed and are briefly described below.

2.3.1.1 Insulin solubility and pH

Normal insulin has an isoelectric point of 5.3, so when it exposed to pH lower than the physiologic pH of 7.4, a decrease in its solubility results, thus reducing its release rate. Langer et al [15, 16] modified insulin with more basic groups to raise its isoelectric point. The resulted tri-lysine insulin had an isoelectric point of 7.4 and thus its solubility increased with a decrease of pH. When incorporating tri-lysine insulin with GluOx in hydrophobic polymers such as ethylene-vinyl acetate copolymer (EVAc), the pH-lowering effect caused by the reaction of GluOx in response to the external increase of glucose led to an increase of the solubility of tri-lysine insulin, thus raising the rate of insulin release.

2.3.1.2 pH-responsive polymer

A pH-responsive polymer is a type of hydrogel that swells or collapses depending on the surrounding pH and can be classified into two categories: anionic and cationic hydrogels, depending on the nature of ionizable moieties on their backbones. Anionic gels usually contain acid groups and swell at high pH and collapse at low pH (unless specified, the pH range discussed in this paragraph is between 7 and 4). When incorporating both GluOx and insulin in a matrix system of anionic gels, the pH-lowering effect from GluOx caused gels to collapse or shrink and consequently enhanced the insulin-release rate due to the "squeezing" action posed by the hydrogel (Figure 2.2) [17]. In another similar design, anionic pH-sensitive material based on acrylic acid was grafted to porous membranes with immobilized GluOx [18, 19] (Figure 2.3). Under low glucose concentration and at pH 7, the polymer chains are expanded and thus they

effectively close the membrane pores. Upon exposure to high glucose, gluconic acid generated by the reaction of GluOx and glucose lowered the pH and resulted in the polymer chains collapsing. Therefore, the pores were open for the diffusion of insulin.

Cationic pH-sensitive hydrogels, usually composed of amine groups, swell at low pH and shrink at high pH. A typical glucose-responsive hydrogel based on the cationic pH sensitive material and GluOx is shown in Figure 2.4 [20, 21]. Under low glucose concentration, the material is in a collapsed state with small pore size that restricts the diffusion of insulin. When exposed to high glucose, the decrease of pH caused by the generation of gluconic acid causes the gel to swell and as a result, increases the insulin release rate.

Based on the expansion properties of the glucose-responsive material composed of cationic pH-sensitive material and GluOx, Siegel and coworkers [22] proposed an implantable "mechanochemical" device as shown in Figure 2.5. The device consists of three chambers: chamber I is composed of the expandable glucose-responsive material just described, chamber II contains aqueous fluid, and chamber III contains insulin solution. When the device is exposed to a higher glucose, the swollen hydrogel generates forces that cause the valve at the chamber III to open up, thus releasing the insulin from chamber III.

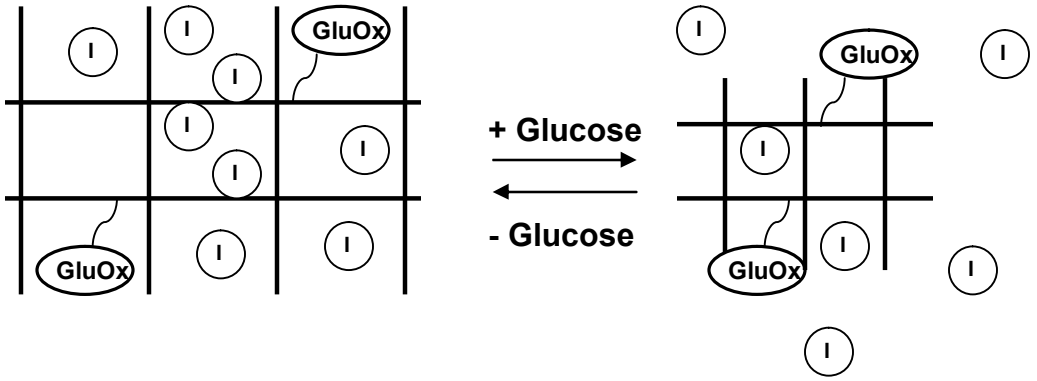


Figure 2.2 Mechanism of glucose-responsive material based on anionic pH-responsive material and glucose oxidase (GluOx). In the absence of glucose, the gel is in a swollen state with the insulin entrapped inside the matrix. In the presence of glucose, the GluOx-glucose reactions cause a decrease in the pH, which leads to a collapse of the hydrogel and "squeezing" out the insulin from the matrix. Figure adapted from [17].

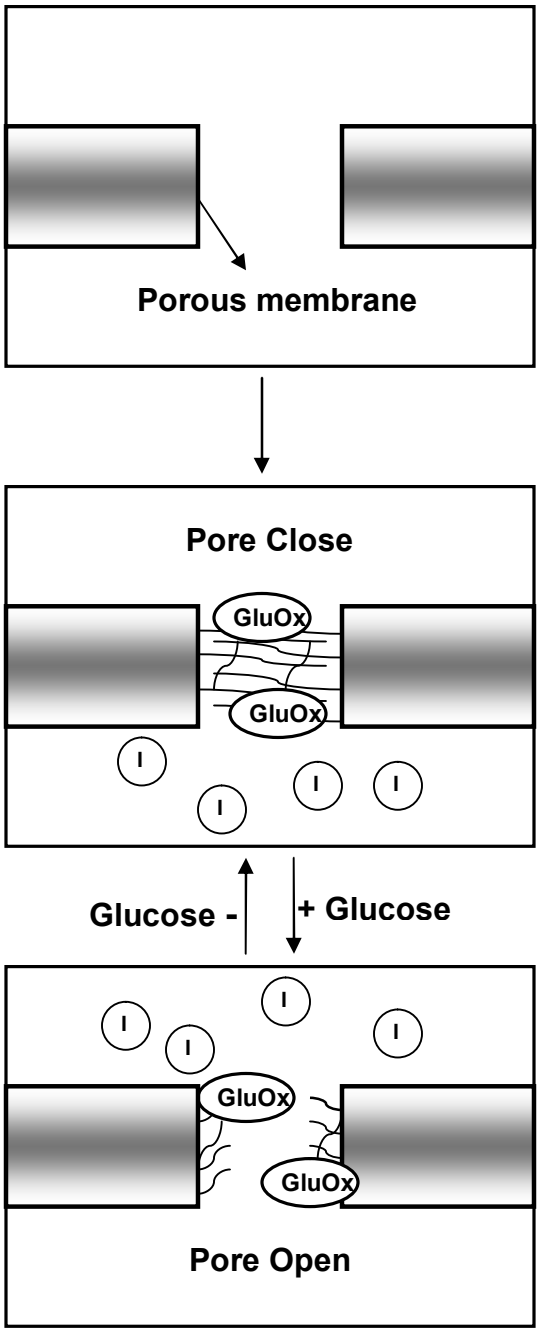


Figure 2.3 Mechanism of glucose-responsive membrane based on anionic pH-responsive material and glucose oxidase (GluOx). Figure adapted from [19]

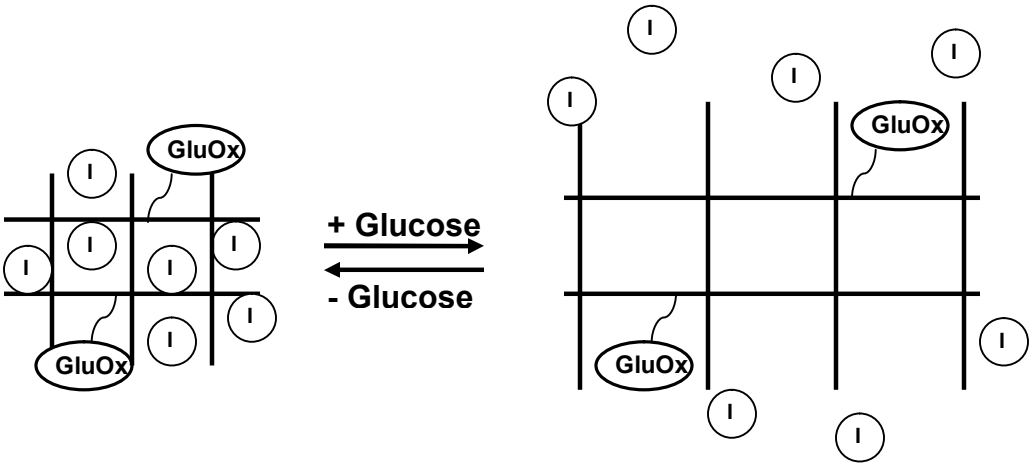


Figure 2.4 Mechanism of glucose-responsive membrane based on cationic pH-responsive material and glucose oxidase (GluOx). Figure adapted from [104]

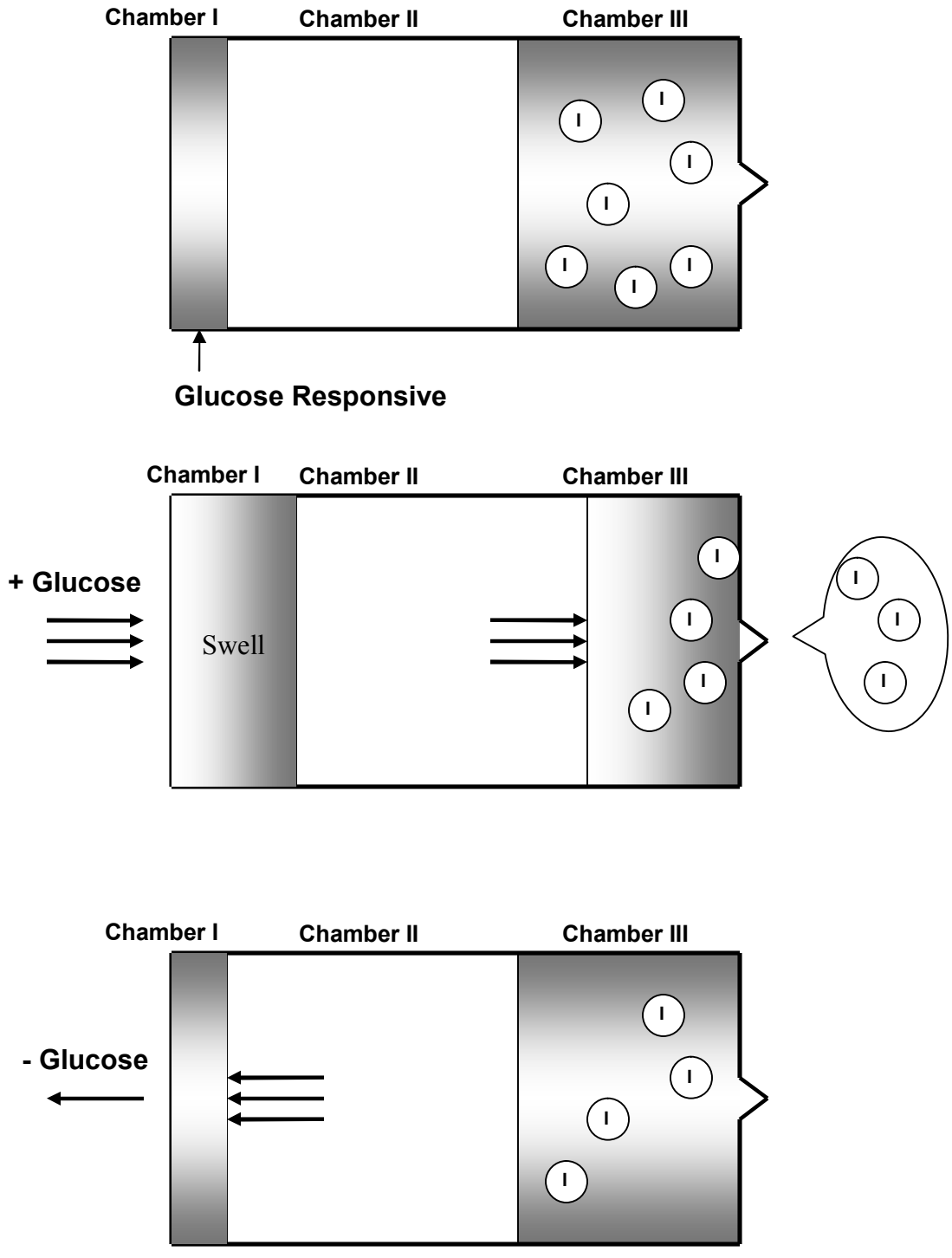


Figure 2.5 Schematic of "mechanochemical" insulin delivery device proposed by Siegel et al [22]. Figure adapted from [105]

2.3.1.3 Polymer erosion

Another approach proposed by Heller [23] is the introduction of a pH-sensitive bioerodible polymer with GluOx. This system is based on the fact that the resultant lowered pH triggered by GluOx can enhance polymer degradation that leads to the release of insulin from the polymer. Another similar approach proposed by Uchiyama et al [24] is to incorporate a phospholipid polymer that would be degraded upon exposure to H_2O_2, the side product of glucose oxidation reaction. Such mechanism is depicted in the Figure 2.6.

The development of glucose-responsive material based on GluOx still faces many challenges. First of all, the stability of GluOx diminishes with time. Of concern also is the production of hydrogen peroxide during the reaction, which has been shown not only to be toxic to the cells but also to deactivate the enzyme. Secondly, the reaction of GluOx and glucose is oxygen limited, hence the decrease of pH is also limited within a small range that could affect the glucose-responsiveness of the polymer. A potential solution involves the incorporation of another enzyme: catalase. Catalase can alleviate the depletion of oxygen and the accumulation of hydrogen peroxide by the following reaction:

$$\mathbf{H_2O_2 \rightarrow 1/2\ O_2 + H_2O}$$

Although adding the catalase into the system improved the properties of GluOx based polymer [25], this type of the material still has major problems of sluggish kinetics in phase changes and is unable to recover to its original conformation after exposing to glucose step changes.

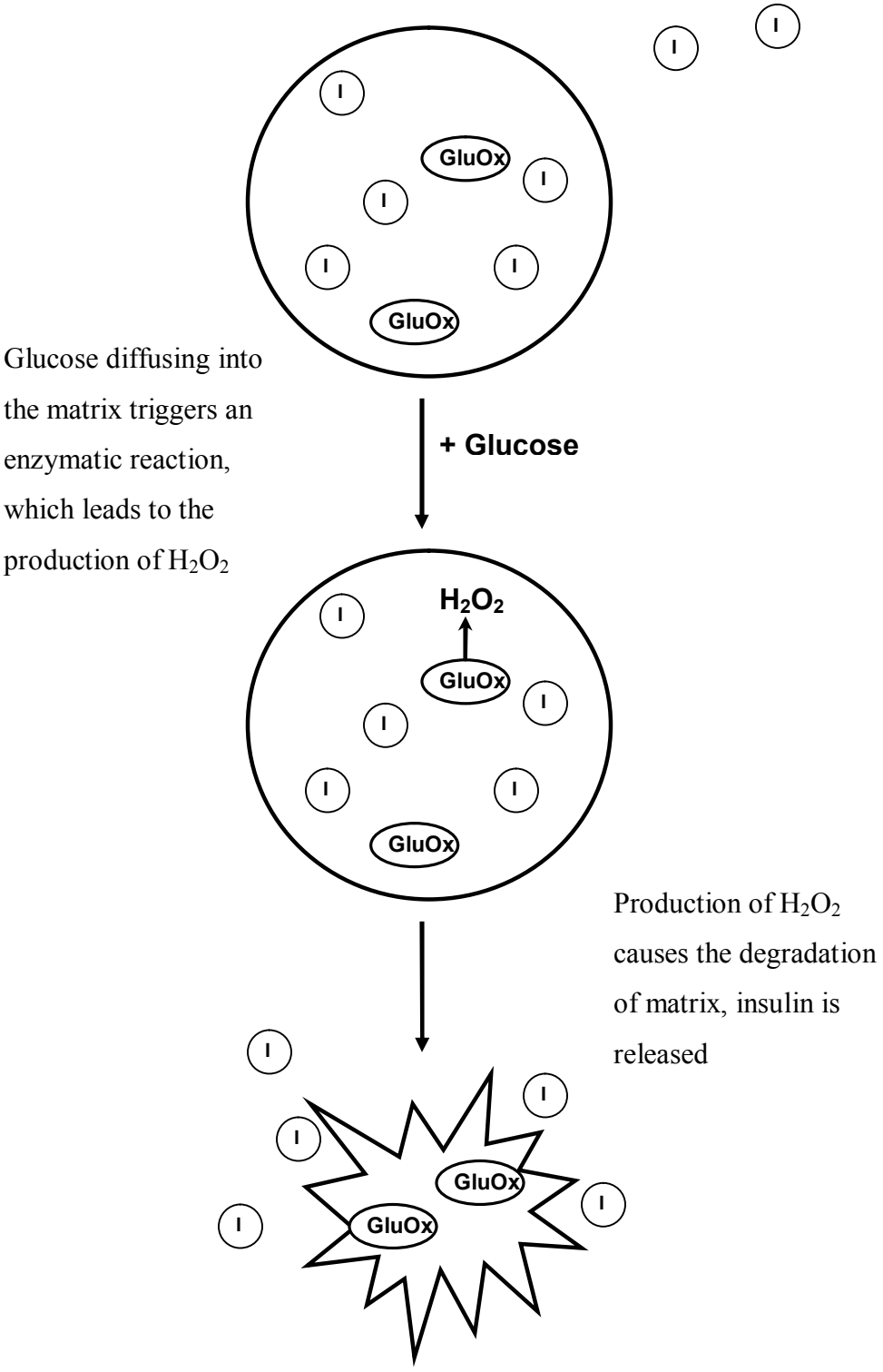


Figure 2.6 Schematic representation of insulin release from glucose-oxidase containing polymer stimulated by glucose. Figure adapted from [24]

2.3.2 Material based on Phenyl-boronate (PBA)

In an aqueous solution, phenylboronic acid and its derivatives have the ability to reversibly bind dihydroxyl molecules such as glucose. The complex between PBA and a polyol compound can be dissociated if another polyol compound is introduced. Based on this unique diol binding property, Shiino et al [26] bound a saccharide-modified insulin (G-insulin) onto a PBA gel column. Because glucose can compete with G-insulin for the PBA diol binding sites, when the column was eluted with glucose solution, the release rate of G-insulin from the column increased relative to the rate detected under glucose-free elution.

PBA can also form a complex gel with a polyol polymer such as poly(vinyl alcohol) (PVA). A copolymer synthesized by Kitano et al [27] from PBA and PVA exhibited glucose-dependent viscosity changes and its mechanism is demonstrated in Figure 2.7. Under low glucose concentration, pendant PBA formed a highly viscous gel with PVA, and insulin was entrapped inside the matrix. When glucose was added to the hydrogel, the gel swelled due to a decrease in the cross-linking density caused by the substitution reaction of glucose with the pendant hydroxyl groups of the polymer toward borate groups. This hence led to the release of insulin from the matrix.

PBA exists in equilibrium between the uncharged and the charged form as shown in Figure 2.8. The complex structure formed by the uncharged borates and glucose is susceptible to hydrolysis, hence it is not stable. As only charged borates can form a stable complex with glucose, adding glucose into the system can shift the equilibrium in the direction of increasing charged phenylborates, which then influence the solubility of the polymer composed of PBA. Based on this glucose-dependent solubility change,

Kataoka et al [28, 29] prepared a copolymer from poly(N-isopropylacrylamide) (PNIPAAm) and PBA, which exhibited a sharp transition in the swelling ratio when exposed to high glucose concentration. On and off regulation of insulin release from such hydrogel was successfully demonstrated with stepwise changes in the glucose concentration.

The advantage of PBA-based polymer is the fact that it is totally synthetic and does not involve a protein that tends to lose its activity with time. Nonetheless, most of the systems described above were functional under physiologically irrelevant conditions such as high pH (above 9) and temperature. To alleviate this, it has been proposed to incorporate amine groups into the PBA-based polymer [30] or to change the chemical structure of PBA [31]. However, since PBA binds to most of the molecules with diol structure, a PBA-based polymer is not highly specific to glucose, and this could be a major concern as there are many molecules containing diol groups *in vivo*.

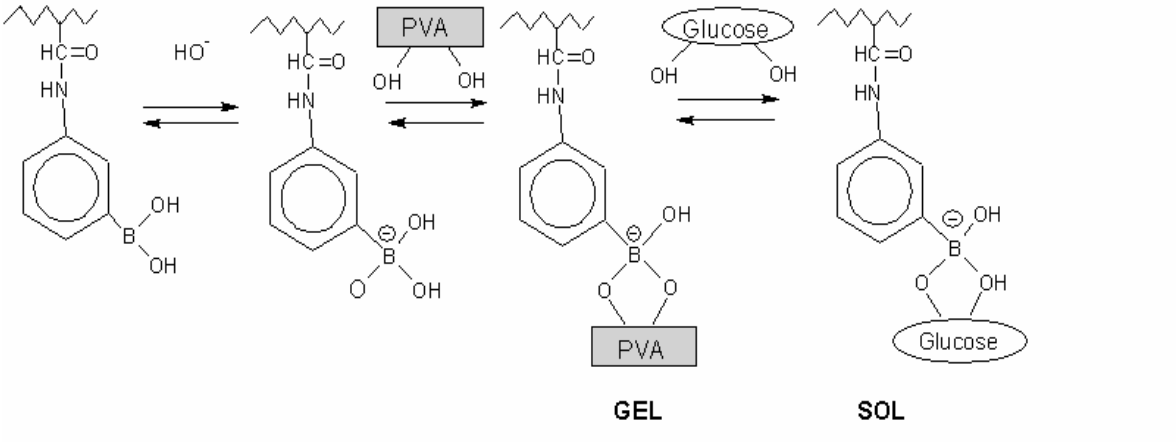


Figure 2.7 Schematic of glucose-responsive material based on PBA and poly(vinyl alcohol) (PVA). Figure adapted from [106]

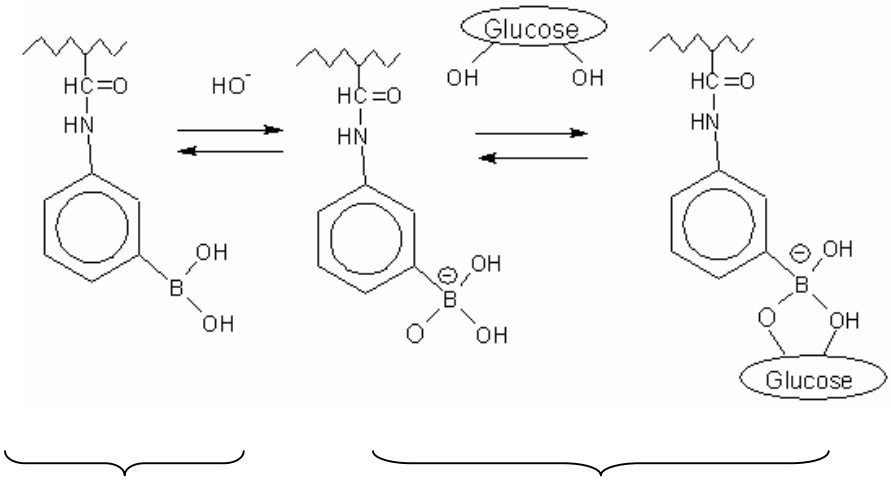


Figure 2.8 Equilibrium of phenylboronic acid. Figure adapted from [104]

2.3.3 Material based on Concanavalin A (Con A)

Con A, a globular plant protein isolated from jack bean, is composed of identical 25,500 molecular weight asymmetric subunits (the amino acid sequence contains 238 residues) arranged in dimeric, tetrameric and higher-order forms. The arrangement of these proteins is affected by the solution properties such as pH, temperature, ionic strength, and salts present in the solution. For instance, at pH 5, most con A molecules exist as a dimer, while at pH 7 con A forms a tetramer. Con A generally binds to saccharides containing α-D-mannose or α-D-glucose residues and appears to recognize terminal as well as internal saccharide residues. The hydroxyl groups that are most critical for binding to con A are situated at positions C-3, C-4, and C-6 of the pyranosyl ring system. Any modification of these hydroxyl groups drastically reduces or abolishes completely interaction with con A. For example, Con A has a very small affinity towards galactose because the orientation of the hydroxyl group at the C-4 position in galactose is different from that in glucose (Figure 2.9) [32].

Due to its saccharide-binding ability, con A has been widely used for purification and characterization of carbohydrates, in the development of glucose-senor, and as a possible candidate for glucose-responsive insulin carrier. Kim et al [33] bound the glycosylated insulin onto con A beads and enclosed those in a polymeric membrane device that is permeable to glucose and glycosylated insulin but not to con A beads. Due to the competitive binding reaction between free glucose and glycosylated insulin towards con A binding sites, exposing the device to high glucose concentration resulted in the release of glycosylated insulin from the device. The release of insulin stopped after glucose concentration was lowered.

Other research groups have focused on the development of glucose-responsive hydrogel by mixing con A with polysaccharides [34-36]. The mechanism is shown in Figure 2.10. Con A, with four glucose-binding sites, functions as a crosslinker for glucose-containing polymer chains, and the mixture of polysaccharides and con A hence forms a viscous hydrogel. In the presence of free glucose molecules, the competitive displacement of the glucose-bearing polysaccharide by free glucose from con A occurs, thus the gel viscosity falls and it becomes sol. Upon the removal of glucose, the sol form of the material is transformed back to gel. The insulin permeability of con A-based hydrogel is thus affected by the presence of glucose. Glucose sensitivity of a con A-based glucose-responsive hydrogel is affected by several factors, including the type of polysaccharide used in the system and any modification of the con A molecules.

A major concern of using con A-based glucose-responsive material is the immunogenicity of con A molecules. Through specific interaction with saccharide-containing cell surface receptors, any leaked con A from the system can cause immune response by activation of T-lymphocytes [37] and also lead to cell death. Moreover, as con A is the glucose-sensing element in the hydrogel, loss of con A would compromise the stability of the hydrogel. Therefore, various groups have proposed different approaches for tethering con A to the polysaccharide chain [38-40], but results thus far are not ideal, such as the glucose sensitivity of the resulting material is not close to the physiologic range.

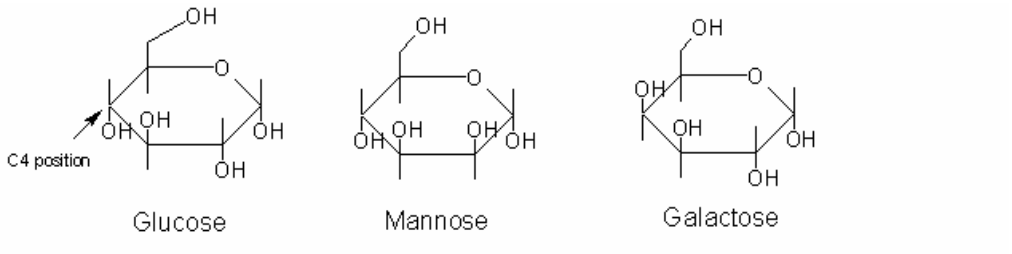


Figure 2.9 Structure of saccharides.

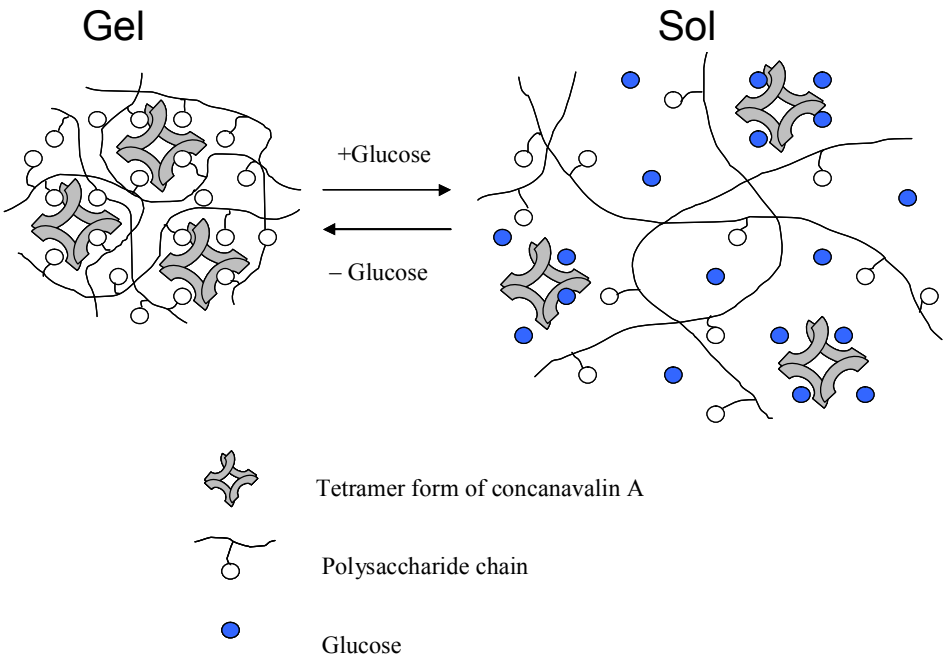


Figure 2.10 Schematic of glucose-responsive material based on con A and polysaccharides.

2.4 Cell based therapy for IDD

2.4.1 Regulation of insulin production and secretion in β cells

Before going into the details of cell-based therapy for IDD, it is important to understand how the β cells regulate their insulin secretion. Figure 2.11 is a simple illustration indicating that β cells use glucose to regulate insulin secretion in a very complex manner from activation of biosynthesis, and processing of the insulin molecules, to the exocytosis of insulin.

At the transcription level, the activation of the insulin promoter requires the binding of a unique set of transcription factors that can only be found in β-cell nucleus, hence, other cell types do not contain the same set of nuclear proteins that can activate transcription [41]. Although the mechanism is not fully understood, glucose does play a key role in the activation of the preproinsulin gene [42]. Total preproinsulin mRNA in the β cells usually remains at a constant level during short-term glucose stimulation (less than 2 hours) [43]. An additional degree of transcriptional regulation can be seen when longer glucose stimulation period (eg. > 4 hours) was implemented. In other words, the increase of proinsulin biosynthesis during short-term glucose stimulation is dominated at the translational level, which includes the recruitment of preproinsulin mRNA from an inert cytosolic pool to translationally active membrane bound polysomes on the rough endoplasmic reticulum, enhancement of mRNA stability, increase in translation initiation and elongation, as well as elevation of biosynthesis of prohormone convertase enzymes PC1/3 and PC2 [43-47].

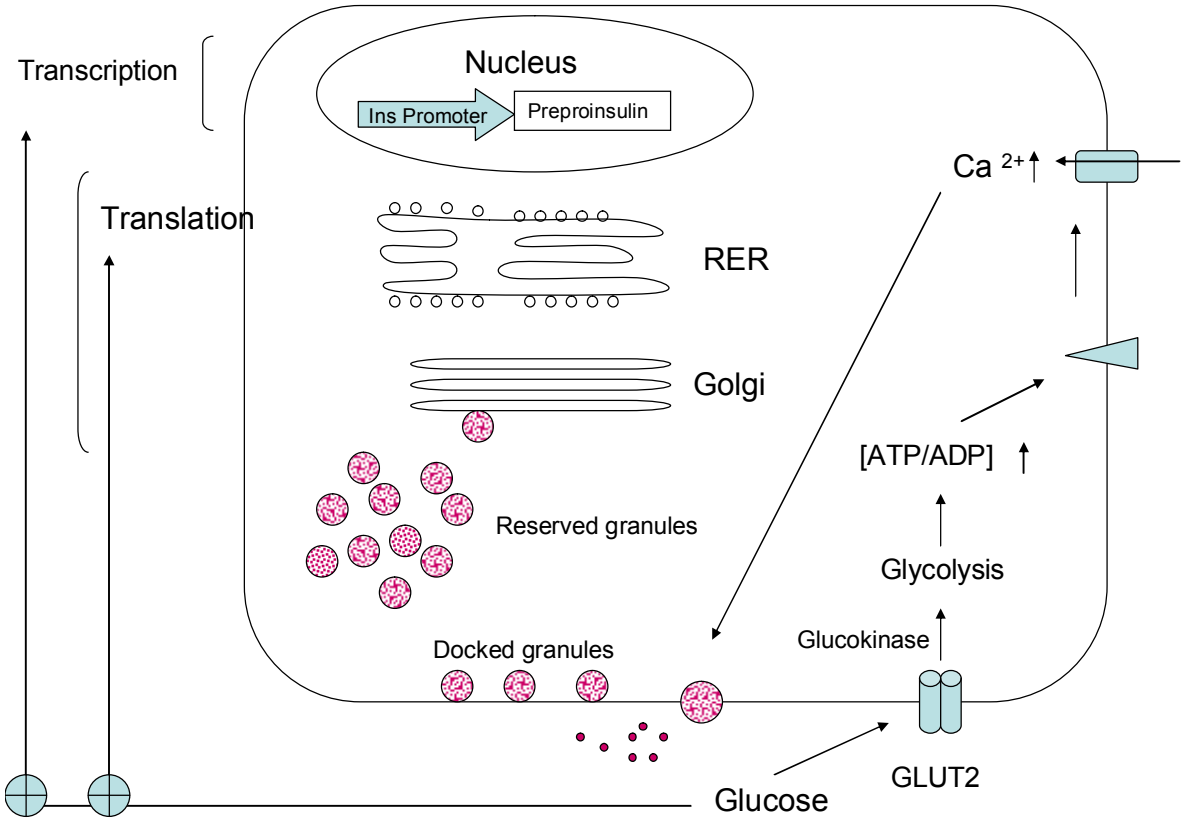


Figure 2.11 Insulin secretion pathways in β cells

After introduction of glucose stimulus, a significant glucose-induced proinsulin biosynthesis can be observed by 20 minutes, reaching a maximum rate by 60 minutes post-stimulate [47, 48]. The off-rate of proinsulin biosynthesis is relatively slow, taking more than 1 hour to return to basal level [48]. Overall, the kinetics of proinsulin biosynthesis do not correlate well with those of insulin secretion from the β cells. Moreover, the threshold concentration of glucose required to stimulate insulin secretion is 4 to 6 mM, but that to stimulate proinsulin biosynthesis is only 2 to 4 mM [49]. Therefore, it is fair to say that the increase of insulin biosynthesis in the cells is to compensate for any loss of insulin by exocytosis, and the fast kinetics of insulin release are determined by the secretory pathway.

When exposed to rapid and sustained glucose stimulation, β cells exhibit a biphasic insulin secretion profile: the first phase is the prompt release of insulin that peaks shortly after stimulation and declines rapidly to a low level; the second phase consists of a gradually increasing rate 30 minutes after glucose stimulation. During the first phase, glucose enters the β cells via high-capacity GLUT2 glucose transporter, which allows fast equilibration of glucose across the plasma membrane. Glucose is then phosphorylated by glucokinase (GK), the first enzyme in the glycolytic pathway. GK has a Michaelis constant K_m of 8 mM for glucose, which explains the concentration dependence of the β cell response to glucose in the physiological range [50, 51]. Further metabolism of glucose leads to an elevation of the ATP/ADP ratio, which causes the closure of K^+ channels and depolarization of β cells [51]. This then activates Ca^{2+} channels, increases the entry of Ca^{2+}, and triggers the exocytosis of the immediately releasable pool of granules. The immediately releasable granules comprise approximately 0.5% of total

granules available, the remaining granules are categorized into morphologically docked (granules that are in contact with the plasma membrane) and reserve granules. These granules are responsible for the second phase insulin secretion, but the mechanism is not fully understood [52, 53].

2.4.2 Islet transplantation

Islet transplantation has become one of the most promising treatments for insulin-dependent diabetes since the development of Edmonton protocol published in 2000. About 80% of the patients who received this treatment maintained insulin-independence with reduced occurrences of hypoglycemia up to a year. The key elements that led to the success of Edmonton protocol include the avoidance of glucocorticoid immunosuppressive drugs that might damage β cells and increase insulin resistance, the optimization of islet function by limiting cold ischemia and immediate transplantation, the usage of sufficient islets isolated from at least two donors, and transplantation of islets into the liver via the portal vein [3]. Despite the exciting outcomes, this protocol is restricted to patients with severe hypoglycemic episodes due to the following two reasons. First, patients who received the islet transplantation are exchanging the burden of frequent insulin injections with the risk of taking lifetime immunosuppressive drugs, which are known to give the short-term side effects of severe mouth ulceration, hypertension, weight loss, anemia, and elevated cholesterol, and their long-term side effects may have not yet been revealed [54]. Second, based on the large patient population and available donors per year in North America, it was estimated that only 0.5% of type 1 diabetes patients can be treated with islets transplantation [55]. This

number was approximated without counting 300,000 new-diagnoses per year in North America and excludes the fact that some patients require more than two islet transplantations. Using islets from other species, such as pig, might solve the availability problem, but the concern of porcine endogenous-retrovirus (PERV) transmission [56] and difficulties in ensuring good-manufacturing production of porcine islets would halt the clinical application. In addition, there is still a long way to resolve the immune-acceptance problems intrinsic to the use of xenografts. Immunoprotection of xenogenic or allogenic cells by encapsulation might reduce the need for immunosuppressive drugs, however, small cytokines can still pass through the semi-permeable membrane and cause cell death; hence it is difficult to achieve complete protection with encapsulation [57]. As the long-term goal of cell-based therapies is to completely correct the diabetic state with unlimited donor sources and without immunosuppressive drugs, a better alternative cell source needs to be sought. Other possible cell sources include: 1) stem cells 2) genetically engineered continuous cell lines and 3) cells of non-β origin. Each of these is described below.

2.4.3 Stem cells

In the area of regenerative medicine, stem cells constitute the most promising cell source because of their ability of self-renewal and the capability of differentiating into different cell types. With limited success due to the lack of complete knowledge in stem cell biology, both human and mouse embryonic stem cells (ES) and adult stem cells have been differentiated into insulin-secreting cells. With adult stem cells, normaglycemia was restored in non-obese diabetic mice that received islet-like cells differentiated from

mice pancreatic ductal epithelial cells [58]. Bonner-Weir et al [59, 60] were able to differentiate the duct tissue obtained after human islet isolation into glucose-responsive, insulin-secreting, islet-like structures. These two findings provide the possibility of using patients' own stem cells to restore their damaged β-cells. However, issues that need to be addressed before considering this approach are as follows: the reported relatively low insulin content in differentiated β-like cells, slow proliferation and differentiation rate with adult stem cells, and most importantly, there is a great chance that the autoimmunity in a type 1 diabetic patient will still recognize the implant and thus immunosuppression or encapsulation will be needed.

When compared to adult stem cell, ES cells have higher proliferation rate and differentiation capacity. Both human [61] and mouse ES [62, 63] cells have been differentiated into insulin-secreting cells either by genetic manipulation or by controlling the culture conditions. Moreover, STZ-induced diabetic mice implanted with mouse ES-derived insulin-secreting cells achieved normoglycemia for up to 16 weeks [64]. Apart from these exciting results, differentiated β-cells from ES cells would possibly encounter the autoimmune rejection in type I diabetes and hence immunosuppression or encapsulation is again required. Furthermore, the potential threat of teratoma formations from the undifferentiated cells within the ES-derived graft [65, 66] and the ethical issues with human ES cells would probably halt the clinical applications of ES-derived cells for a long time.

2.4.4 Continuous cell lines

Because of the availability issue with islet transplantation, and the difficulties in

using cells derived from stem cells, one of the promising approaches is to expand β cells through the development of continuous β cell lines. Efrat et al [67] have developed a family of continuous β cell lines by expressing the SV40 large T-antigen (Tag) under the control of the insulin promoter in transgenic mice. One of the early cell lines, βTC3, can be propagated easily in culture, secretes high amounts of insulin, and exhibits a secretion profile similar to that of mouse islets (See Chapter 3). However, due to its high expression of GLUT1 instead of the GLUT 2 glucose transporter and of hexokinase instead of glucokinase, βTC3 cells are hypersensitive to glucose and would not be applicable for implantation [68]. Later cell lines such as βTC6 were able to secrete insulin in response to physiologic glucose concentrations, but their ability to secrete insulin diminished with time in culture [69]. Due to the fact that increasing passage number of continuous β cell lines could cause them to lose insulin secretion properties, and also as one needs to prevent overgrowth *in vivo*, another cell line, βTC tet, was developed to allow control over the growth [70]. The expression of Tag gene in such cells was under control of the regulatory elements of the bacterial tetracycline (tet) operon: in the absence of tetracycline, βTC-tet can be propagated easily in culture; in the presence of tetracycline, cells undergo growth arrest with an increase of insulin secretion rate. βTC-tet cells also exhibit a secretion profile similar to islets (see Chapter 2) and display proper glucose-responsiveness. Animal studies with βTC tet cells have shown some promising results in restoring normoglycemia (Weber et al, data not published).

Developing human insulinoma cell lines has proven to be more challenging than anticipated. Even if one successfully develops a human cell line with proper insulin

secretion characteristics, immunoprotection of these cells through cell-encapsulation in semipermeable membranes is still a must to prevent graft failure intrinsic to the use of allogeneic cells and possible autoimmunity towards β cells, and concern of tumorigenic phenotype of human β cell line.

One of the promising approaches is to encapsulate these cells in alginate/poly-L-lysine/alginate (APA) microbeads. Studies with βTC3 and βTC-tet without growth suppression have shown that cells continued to grow post-encapsulation, and the growth pattern inside the microbeads depended on the alginate composition and oxygen availability. For instance, with βTC3 cells, no significant growth suppression occurred in beads made of high mannuronic (M) alginate, but a prolonged period of growth suppression was observed if the alginate was of high guluronic (G) acid content [71]. Although freshly encapsulated βTC cells exhibit a secretion profile very similar to unencapsulated cells, it is unknown whether this remodeling process and growth inhibition would cause any changes in the insulin secretion profile, and we attempted to address this question in Chapter 3 of this thesis.

2.4.5 Genetically engineered non-β cells

Non-β cells can be taken as a biopsy from a patient and be genetically engineered *ex vivo* to become insulin-secreting cells. Genetically modified cells with the proper insulin-secretion characteristics could then be directly implanted through engraftment or be associated with biomaterials in a construct, the latter having the advantage of retrievability if needed. Since these cells are autologous and not β-cells, they relax the immune acceptance problems intrinsic to the use of xeno- or allogeneic cells as well as

the autoimmunity towards β cells in Type 1 diabetes. Presumably, these cells have no supply limitations and can be cryo-preserved after genetic modification for subsequent needs.

To be eligible as a potential replacement of daily insulin injections, β-cell surrogates must possess two important features: the ability to process and produce mature insulin and to release insulin kinetically coupled with changes in blood glucose concentrations. In terms of producing mature insulin, because most non-β cells do not carry the two prohormone convertases PC1/3 and PC2, which cleave the proinsulin at the B-C junction after Arg-32 and the C-A junction after Arg-65, respectively, the two processing sites of the proinsulin molecule were converted by Yanagita to a consensus furin recognition site [72-74]. As furin is ubiquitously expressed in most cells, non-β cells such as hepatocyes, fibroblasts, and myoblasts that were transfected with the modified preproinsulin gene can produce functional insulin. An additional mutation was later introduced to increase the stability of the insulin molecule by changing histidine B10 to aspartic acid [72]. The preproinsulin cDNA used in CHAPTERS 5, 6 is furin compatible with B10 mutation. Another approach proposed by Lee et al [75] to equip cells with the ability to produce bioactive insulin was to construct a single-chain insulin analogue without the need of enzymatic processing. However, the recombinant modified insulin has only 20-40% activity of human insulin, and there is the concern of possible physiological abnormalities caused by the lack of C-peptide [76].

Although non-β cells now have the ability to produce and secrete mature insulin, the major obstacle lies in coupling the synthesis and release of recombinant insulin to glucose concentrations. The following paragraphs discuss specific approaches taken

with different cell types as the insulin source and their rationale.

2.4.5.1 Hepatocytes

Liver is the first main organ that insulin reaches via the blood stream and is also the major site of insulin action; hence, the most popular cell type to be developed into a β surrogate is the hepatic cell. Besides the above reasons, hepatocytes possess glucose-sensing ability because they express GLUT2 [77] and glucokinase [78], the two important glucose-sensing elements existing in β cells. Moreover, hepatocytes not only regulate the expression of various proteins but also express some genes under glucose-responsive transcriptional control that can be utilized to regulate insulin secretion. Examples described below are the promoters for phosphoenolpyruvate carboxykinase (PEPCK), Glucose-6-phosphatase (G6Pase), and L-type pyruvate kinase (L-PK).

The phosphoenolpyruvate carboxykinase (PEPCK) promoter is regulated in a complex manner by insulin, glucagon, and glucose. Insulin expression under the control of PEPCK is up-regulated by glucagon (via cAMP) and glucocorticoid and down-regulated by insulin [79]. Nonetheless, there exists evidence that the promoter might be suppressed by glucose independently of insulin, hence, this promoter might not be ideal for clinical application [80, 81]. G6Pase gene promoter is stimulated by glucose and suppressed by insulin [6]. This promoter is relatively weak and was modified to enhance its activity by incorporating the liver specific aldolase B enhancer in the promoter region. Adenoviral delivery of insulin gene under the regulation of modified G6Pase promoter into STZ-induced diabetic rats has shown some promising results, but the glucose-challenge tests indicated sluggish kinetics of insulin release [82].

The L-PK promoter is the regulatory element in controlling the expression of the liver-type pyruvate kinase gene in response to glucose. However, the promoter was believed to be relatively weak and unlikely to induce efficient transgene expression. Nonetheless, Lee et al [75] demonstrated that when the L-PK promoter was used to express a single-chain insulin analog (SIA) with an SV40 enhancer to elevate the basal levels of SIA expression, in the setting of recombinant adeno-associated virus (rAAV) that were later injected into the portal vein, there was sufficient insulin to restore normoglycemia in diabetic rodents. Secretion of SIA from hepatocytes was indeed glucose-dependent, but glucose-tolerance tests showed there was a 3-4 hour delay of SIA peak level after administration of glucose and 6 hours at minimum for SIA to return to basal level after removal of glucose. Thule et al [83, 84] connected the glucose-responsive element (GIRE) from the L-PK promoter into the liver-specific, insulin-suppressive basal promoter elements from the insulin-like growth factor binding protein-1 (IGFBP-1). Insulin secretion from this cassette was glucose up-regulated and insulin down-regulated. Using adenovirus as the delivery vehicle by injection into the portal system of spontaneously diabetic BB/Wor rats, the transgene restored normoglycemia up to 12 weeks [85].

Although utilizing glucose-responsive promoters enables the hepatic cells to release insulin in response to glucose concentrations, the kinetics of insulin release from these cells are slow compared to the rapid insulin release from the β cells. This is largely due to the fact that hepatocytes do not carry secretory granules and insulin secretion is regulated only at the transcriptional level. Even if transcriptional responses can be improved, pre-existing mRNA can result in continued production of insulin.

Consequently, the sluggishness of insulin secretion might result in the patient experiencing a period of hyperglycemia prior to transcriptional activation and secretion of insulin; and a period of hypoglycemia before the glucose-responsive promoter is down-regulated and all the insulin mRNA degraded. To expedite the degradation of mRNA thus improving the down-regulation kinetics, Tang and Sambanis [86] engineered the preproinsulin mRNA so as to destabilize it through nonsense-mediated mRNA decay (NMD). The engineered preproinsulin mRNA carried three insulin gene copies with stop codons in the middle of the mRNA molecule to induce NMD. The level of the engineered preproinsulin mRNA expressed under transcriptional regulation in human hepatomas declined faster upon switching off transcription than that of the one-copy non-engineered control. This methodology is expected to be generic and applicable to different host cells, including primary cells.

2.4.5.2 Myoblasts

Myoblasts is another attractive cell choice for insulin as well as other therapeutic protein delivery. Because skeletal muscle constitutes a high percentage (40%) of body weight of an average individual [87], myoblasts are abundant and easily accessible. When growing *in vitro*, myoblasts are dividing rapidly and can be genetically engineered to secrete high amounts of recombinant proteins. These cells also have the potential of differentiating into non-dividing myotubes, which can survive for long periods of time *in vivo* if properly implanted. Types of therapeutic protein that have been expressed in myoblasts include: ciliary neurotrophic factor (for neurodegenerative disease) [88], erythropoietin (for chronic renal failure, cancer or AIDS-associated anemia) [89], factor

IX (for haemophilia B) [90, 91], factor VIII (for haemophilia A) [92], growth hormone (for skeletal muscle wasting) [93], and vascular endothelial growth factor (for promoting localized angiogenesis) [94].

Genetically modified myoblasts can be injected into muscle tissue for therapeutic use, however, this approach faces some drawbacks. One is the decrease of cell viability observed to occur few days after injection. A second is that the long-term survival of the implant depends on the rare fusion events with host muscle cells. A third is the concern of retrieveability if needed. Hence, it appears necessary to associate myoblasts with biomaterials prior to implantation. One of the promising approaches proposed by Aebischer et al [88, 89] is to encapsulate myoblasts in polyethersulfone hollow fibers and later induce myoblast differentiation by culturing the fibers in differentiation media prior to implantation. Another approached proposed by Vandenburgh et al [93] is to tissue-engineered myoblasts into a bioartifical muscle (BAM). BAM was developed by suspending proliferating myoblasts in an ECM gel and then casting the mixture into silicon rubber molds containing end attachment structures (artificial tendons). After the gel solidified and dehydrated, the resulting cylindrical structure caused the differentiating muscle fibers to organize into parallel bundles. Although the morphologic features of the BAM are significantly different from those of *in vivo* skeletal muscle, BAM has the advantages of retrieveability and long-term usage as a living protein delivery device. In contrast to the approaches mentioned above, myoblasts encapsulated inside alginate do not proliferate as well and the ability of differentiation seems to decrease [95].

In terms of insulin delivery, when modified preproinsulin cDNA that is furin compatible was transfected into myoblasts, both undifferentiated myoblasts and

differentiated myotubes can process proinsulin into active human insulin with high efficiency and high secretion rate [7, 96]. However, because these cells lack a regulated secretory pathway as well as the glucose sensing elements, the insulin secretion characteristic will never meet the clinical requirements. Due to the advantage of a living protein delivery device mentioned above, researchers now suggest to use engineered myoblasts in providing basal insulin levels along with fast–acting insulin preparations to cover meals.

2.4.4.3 Fibroblasts

Fibroblasts have the same advantages and disadvantages as myoblasts in developing insulin-secreting cells for IDD treatment. Instead of regulating insulin release at the transcription level, Rivera et al [97] proposed a novel approach, in which they used a human fibrosarcoma cell line HT1080 as the model, to regulate insulin secretion in the endoplasmic reticulum (ER). In their system, furin-compatible proinsulin was expressed as a fusion protein with a conditional aggregation domain that interacted with itself in a ligand-reversible manner. In the absence of the ligand, aggregates of proinsulin were too large to depart the ER and were retained within the organelle. When the cells were exposed to a cell-permeant ligand, FKBP12, aggregates of proinsulin dissolved, the aggregation domain and C-peptide were removed by furin in the trans-Golgi apparatus, and insulin was released from the cells. The kinetics of insulin release were relatively fast compared to transcriptionally regulated cells, the highest insulin-secretion rate was observed 30 minutes after drug administration, and the rate declined to 10 to 20% of peak level 2 hours after the removal of the stimulus. When

cells expressing proinsulin fusion protein were implanted into STZ diabetic mice, intravenous administration of the ligand into the animal helped to bring down the blood glucose within 2 hours. The major drawback of this approach is that the release of insulin was not regulated by glucose but by a synthetic drug that might be harmful to the patient after long-term use, and it is unclear whether accumulation of protein inside the ER would cause some toxic effects to the cells. Nonetheless, this approach indeed successfully demonstrated the possibility of regulating insulin release at the post-translation level within the cells.

2.4.4.4 Non-β Endocrine Cells

Since the main reason that genetically-engineered nonendocrine cells such as hepatocytes, myoblasts, and fibroblasts, can not exhibit proper secretion profile is because they lack the secretory pathway to secrete insulin in response to glucose, it is reasonable to think that using cells expressing a regulated secretory pathway might solve the problem. The AtT-20 cell line, derived from an anterior mouse pituitary tumor, is the first neuroendocrine cell being engineered to become β surrogate because it contains both the constitutive and regulated secretory pathways, has enzymes to process proinuslin into insulin, and expresses glucokinase (but not GLUT 2) [98] so it might be able to exhibit glucose-responsive secretion. Studies have shown that overexpression of GLUT2 and glucokinase enabled insulin-secreting AtT-20 to release insulin in response to glucose, however, with a maximal rate at the subphysiological glucose concentration of 10 μM [99], and in addition, the manipulation resulted in glucose-induced toxicity and severe apoptosis in these cells [100]. Another concern of using this cell type is that they

also have high production of endogenous adrenocorticotropic hormone (ACTH), which would upset the hormonal balance of the patient and cause serious disease (Cushing's syndrome). Futhermore, it might be difficult to obtain and transfect the autologous pituitary cells.

The secretion kinetics of incretin hormones, glucagons-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), from two intestinal enteroendorcine cells, L-cells and K cells, are very similar to those of insulin from β cells following glucose ingestion by normal human subjects [101]. Based on this unique property, Cheung et al [102] expressed proinsulin in transgenic mice under control of the GIP promoter. The results showed that the transgene expressed specifically in K cells of duodenum and stomach, and after the destruction of β cells, STZ diabetic mice were able to regulate glycemia and exhibit a proper response to glucose tolerance test. Tang and Sambanis [103] genetically engineered a human intestinal enteroendocrine L-cell model, the NCI-716 cell, by recombinant adeno-associated virus serotype 2 (rAAV2), and showed that the differentiated L cells released recombinant insulin simultaneously with endogeneous GLP-1 upon stimulation. This research has opened significant avenues for cell-based therapy in treating insulin-dependent diabetes, but clearly more studies need to be done, including translating this approach from transgenic embryos to adult animals.

2.5 Cell-material hybrid constructs

As discussed above, while various glucose-responsive materials have been developed and different cell types have been genetically engineered for regulated insulin release, the two have not yet been combined in a hybrid construct. In such a device,

cells can act as continuous insulin secretor and the material can act as a control barrier for insulin released by the cells. The concept and proposed prototype are addressed in this thesis.

2.6 Objective of the thesis

As discussed above, a major concern in selecting a proper cell source for developing a tissue engineered pancreatic substitute is the secretion properties of the cells. Thus, the overall objective of this thesis is to characterize and improve the secretion properties of tissue engineered pancreatic substitutes based on genetically engineered cells, and the specific aims are: (1) to characterize the effects of cell remodeling process on the secretion properties of the encapsulated insulinoma cells (CHAPTER 3); (2) to develop a new methodology for improving the secretion properties of genetically engineered non-β cells, namely, using a glucose-responsive material based on con A to control the release of insulin secreted by the cells (CHAPTERS 4 and 5); (3) to improve the glucose sensitivity of the con A-based material so that it responds to physiologically relevant glucose concentrations (CHAPTER 6). Conclusions and future directions for each specific aim are described in CHAPTER 7.

CHAPTER 3

INSULIN SECRETION DYNAMICS OF FREE AND ALGINATE-ENCAPSULATED INSULIN-SECRETING CELLS

3.1 Abstract

In developing a pancreatic substitute for the treatment of insulin-dependent diabetes, a major constraint is the limited availability of normal pancreatic islets. This constraint can be relaxed by using genetically engineered continuous β cell lines, which grow easily in culture and also possess some of the insulin secretion characteristics exhibited by normal β cells. To improve their immune acceptance, cells can be encapsulated in alginate/ poly-L-lysine/ alginate (APA) beads without loss of secretory capacity. However, as a result of cell growth and death, there is remodeling in the capsules, and this may change the secretory dynamics of the encapsulated cell system. In this study, we cultured mouse βTC3 insulinomas in APA beads made of high guluronic (G) or high mannuoric (M) alginate, and conditionally transformed mouse βTC-tet insulinomas in high M alginate beads in the absence of tetracycline or in the presence of the antibiotic to suppress cell growth. The secretion dynamics of the preparations were characterized at various time points by loading beads in a single-pass perfusion system and performing step changes in the concentration of glucose in the perfusion medium. Secretory profiles were compared against free cell controls and free porcine pancreatic islets. Results indicated that the insulin secretion profile changes as the beads remodel with time in culture, however, the secretion profile can be retained if cell growth is suppressed. The implications of these findings regarding *in vivo* experiments with diabetic animal

models are discussed.

3.2 Introduction

The treatment of type 1 insulin-dependent diabetes by transplantation of allogeneic pancreatic islets is receiving increased attention following the success of the Edmonton protocol [107]. However, a major limitation of this approach is the shortage of human islets from cadaveric donors. Additionally, the steroid-free immunosuppression used, although more benign than conventional regimens based on cyclosporine, still results in long-term complications [108, 109].

To overcome these problems, cell sources alternative to human islets and methods to reduce the necessary immunosuppression are being sought. For the latter, encapsulation in permselective membranes is commonly employed to provide partial immune protection of an implant from the host. Cells from a different species, e.g., porcine islets, are theoretically amply available. However, there exist significant technical challenges associated with their large-scale isolation under the required conditions of purity and sterility, and concerns about the possible transmission of pathogens to humans remain [56]. Furthermore, survival and function of xenografts in non-immunocompromised hosts is particularly difficult to accomplish even with encapsulation [57, 110, 111]. The family of βTC insulinomas developed in transgenic mice by Efrat and co-workers constitutes an important development towards solving the cell availability problem [67, 70]. These cells are easy to amplify in culture, they have a high insulin secretion rate relative to other continuous β cell lines or genetically engineered non-β cells, and they retain many important insulin secretion characteristics of normal β cells. The βTC-tet

cells in particular, in which expression of the SV40 T antigen (Tag) oncoprotein is tightly and reversibly regulated by tetracycline, proliferate when Tag is expressed in the absence of tetracycline and stop proliferating in the presence of tetracycline that shuts off Tag expression [70, 112]. Such reversible transformation is an elegant approach in generating a supply of β cells via proliferation of an inoculum, followed by arrest of cell growth when the desirable population size is reached. Although human cell lines similar to βTC are not currently available, they may be developed in the future. Furthermore, encapsulated βTC lines constitute an important model of a pancreatic substitute for studies *in vitro* and in diabetic animal models *in vivo*.

As the βTC3 and, in the absence of tetracycline, the βTC-tet are proliferative cell lines, the cells continue to grow post-encapsulation. The growth pattern exhibited in the commonly used calcium alginate/ poly-L-lysine/ alginate (APA) beads depends on the alginate composition [113-115] and is also influenced by oxygen availability [116]. For instance, βTC3 cells in APA beads experience prolonged growth suppression if the alginate is of high guluronic (G) acid content, whereas no significant growth suppression occurs in beads made of high mannuronic (M) alginate. Following growth-mediated remodeling, the cells form clusters, which are distributed differently in high G and high M-based beads. Although APA-encapsulated βTC cells retain for prolonged periods of time their capacity to secrete insulin, the precise dynamics of insulin secretion upon glucose up and down-regulation and the effect of growth-mediated remodeling on secretion dynamics remain unknown. Insulin secretion dynamics are important at restoring normoglycemia *in vivo*, especially in higher animal models.

In this study, βTC3 and βTC-tet mouse insulinoma cells were encapsulated in APA

beads of different alginate compositions and propagated for prolonged periods of time. The insulin secretory profiles in response to glucose concentration step changes were evaluated at different time points using a single-pass perfusion system. The effect of tetracycline on the secretory profile of encapsulated βTC-tet cells was also characterized. Results were compared against those of free βTC cells, free porcine, and one preparation of free human pancreatic islets. The implications of these studies to *in vivo* experiments are discussed.

3.3 Materials & Methods

3.3.1 Cells and cell culture

βTC3 [67] and βTC-tet [70] cells were obtained from the laboratory of Shimon Efrat, Albert Einstein College of Medicine, Bronx, NY. Cells of passage number 42-45 (βTC3) and 33-37 (βTC-tet) were used in this study. Cells were cultured as monolayers in T-flasks with culture medium changed every 2-3 days. Culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis MO) with 25 mM glucose, supplemented with 15% horse serum (from Hyclone (Logan, UT) and Sigma for βTC3 cells and βTC-tet cells respectively), 2.5% bovine serum (Mediatech, Herndon, VA), 1% penicillin-streptomycin (Mediatech) and L-glutamine (Gibco, Grand Island, NY) to a final concentration of 6 mM. Upon reaching 100% confluence, cells were harvested by trypsin-EDTA (Sigma) for encapsulation.

Porcine islets were isolated in Dr. Hering's lab at the University of Minnesota. Islets were cultured overnight, then shipped by overnight express mail to Drs. Weber and Safley at Emory University, Atlanta, Georgia. Upon arrival in Atlanta, a fraction of the

islets were transferred to Georgia Tech for immediate initiation of a perfusion experiment. Thus, perfusion experiments were performed 2 days after islet isolation. Human islets were isolated a day prior to the perfusion experiment from a cadaver by the Emory University Juvenile Diabetes Research Foundation International Center for Islet Transplantation.

3.3.2 Cell encapsulation

Cells were encapsulated at a density of $3x10^7$ cells/ml alginate using an electrostatic droplet generator (Nisco Engineering Inc., Zurich, Switzerland) and following the procedure of Stabler et al [114] for preparation of alginate-poly-L-lysine-alginate beads. Low molecular weight, high guluronic acid content alginate (LVG: 73% guluronic content) or low molecular weight, high mannuronic acid content alginate (LVM: 38% guluronic content) (Pronova, Norway) were used for βTC3 cells; high mannuronic acid content alginate (40% guluronic content) (ISP Inc., San Diego, CA) was used for βTC-tet cells. The final bead size was 700-800 μm in diameter. Bead aliquots of approximately 2 ml volume were cultured with 40 ml of culture medium in each of five T-75 flasks placed on an orbital shaker within a humidified 37°C, 5% CO_2/95% air incubator. The spent medium was completely replaced with the fresh medium every two to three days at the beginning of the experiment and every one to two days towards the end as cells grew. For the encapsulated βTC-tet cells, beads were propagated without tetracycline for 20 days, at which point the culture was split in two: one-half continued to be propagated in the absence of tetracycline, and the other half was propagated in the presence of 30 ng/ml tetracycline (Sigma) up to day 30. For all

entrapments, one of the T-75 flasks was used to withdraw samples of beads for perfusion and histology examination, while the others were used for glucose concentration measurements. Three independent (n=3) bead preparations were performed for each type of culture studied (βTC3 cells in LVG, βTC3 cells in LVM, and βTC-tet cells in M alginate).

3.3.3 Perfusion system and experiments

The configuration of the perfusion system is shown in Figure 3.1. A spindle-shaped glass tube of approximately 0.34 ml volume was used as the cell chamber. Cotton was placed at both ends of the chamber to retain the cells, free or encapsulated. A peristaltic cassette pump (Manostat, New York, NY) was used to generate the required flow rate; a multiport valve positioned before the cell chamber was used to control the type of medium flowing through the bed. The whole apparatus except for the fraction collector was placed within a humidified 37°C, 5% CO_2/95% air incubator. Effluent samples were stored at -20^{o}C for later assay of insulin. The flow properties were evaluated by flowing through a cell-free system a square wave of FITC-insulin (Sigma) in PBS and assaying the FITC-insulin in effluent samples.

With free βTC3 and βTC-tet cells, freshly trypsinized cells were counted by trypan blue (Sigma). A total of $6x10^6$ cells were mixed with macroporous cellulose (Sigma) and gradually loaded into the cell chamber. With encapsulated βTC3 and βTC-tet cells, approximately the same number of cells, corresponding to 0.2 mL of alginate beads, was loaded in the perfusion chamber without any cellulose. Cells were perfused at a flow rate of 0.25±10% ml/min, which ensured differential operation of the bed and measurable

insulin concentrations in the effluent samples. Each experiment started by flowing culture medium for 30 minutes to allow for cell recovery from the loading process. Basal medium consisting of glucose-free DMEM without glutamine (Sigma) was then flowed through the bed for 1 hour to bring cells to their basal secretion level. A glucose square wave of 1.5 hours high glucose was then implemented followed by 1.5 hours of basal medium. The high glucose medium consisted of DMEM without glutamine and with 8 mM glucose for βTC3 or with 20 mM glucose for βTC-tet cells. For βTC-tet cells propagated in the presence of tetracycline, all perfusion media were supplemented with 30 ng/ml tetracycline.

With porcine or human islets, approximately 1000 IEQ were mixed with cellulose and loaded into the perfusion chamber. The perfusion medium used for 30 min of islet conditioning consisted of E199 (Mediatech, Inc., Herndon, Virginia) containing 5.5 mM glucose and supplemented with 10% heat-inactivated porcine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.2% ciprofloxacin. RPMI medium (Sigma) supplemented with BSA (Sigma), HEPES (Sigma), and glucose at concentrations of 1.67 mM and 16.7 mM was used for basal and induced conditions, respectively.

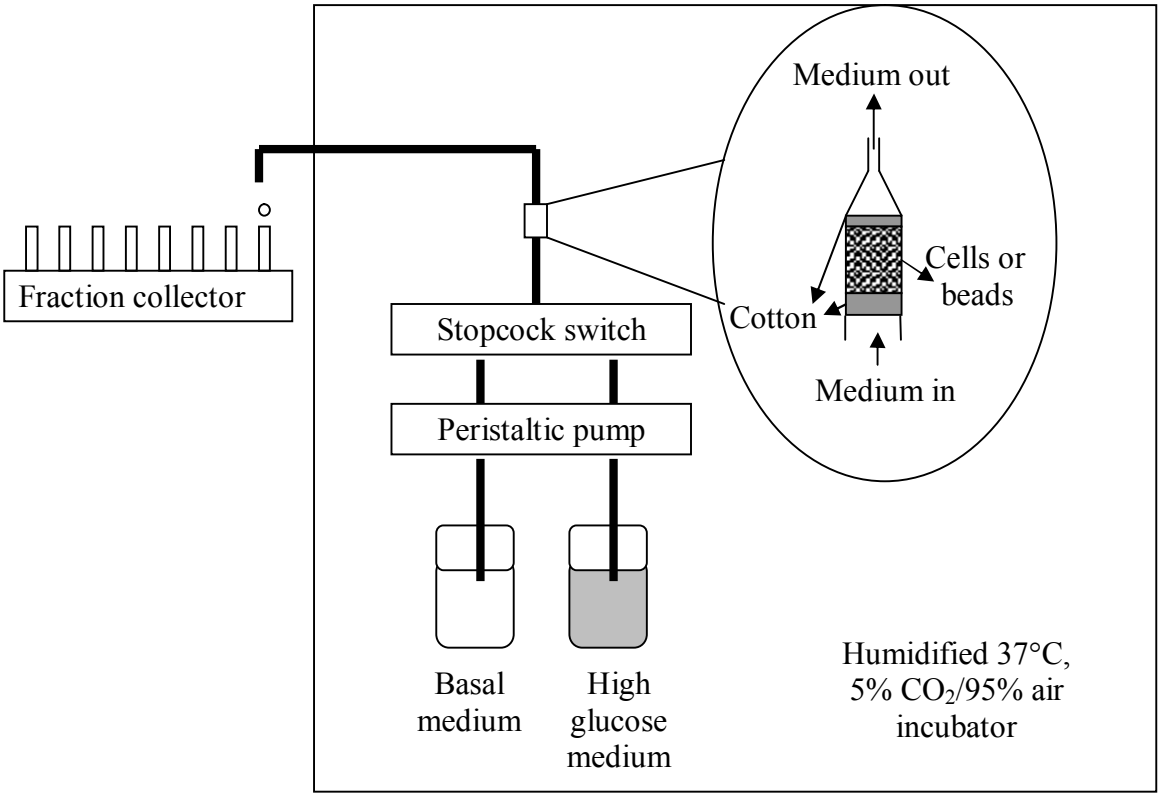


Figure 3.1 Single-pass perfusion system for measuring the secretion dynamics of free and encapsulated cells subjected to step changes in glucose concentration. Free cells were mixed with macroporous cellulose and then gradually loaded into the cell chamber. Both free and encapsulated cells were retained in place with cotton plugs positioned upstream and downstream of the bed. Step changes in glucose concentration were implemented by switching from one medium to the other using a multiport valve. Effluent samples were collected in a fraction collector for subsequent assay of secreted insulin by a rat insulin radioimmunoassay.

3.3.4 Assays

FITC-insulin concentrations were measured by a fluorescence plate reader (Spectra Max Gemini Plate Reader, Molecular Devices Corp., Sunnyvale, CA) using a standard curve constructed from known FITC-insulin concentrations. Glucose concentrations were determined by Glucose Trinder assay (Diagnostic Inc, Oxford, CT). Secreted insulin was assayed by a rat insulin radioimmunoassay (Linco Research, St. Charles, MO), in which the insulin antibody exhibited 100% cross-reactivity against mouse insulin (manufacturer's data). Numbers of βTC3 and βTC-tet cells were measured by mixing suspensions of trypsinized cells with the trypan blue (Sigma, St. Louis, MO) and counting cells on a hemocytometer.

3.3.5 Histology

For histology, beads were washed with 1.1% $CaCl_2$ and fixed in 3 % glutaraldehyde (Sigma). After embedding in a paraffin block, beads were sectioned, and slides were stained with hematoxylin/eosin (H/E).

3.4 Results

3.4.1 Control experiments

To evaluate the time lag introduced by the free volume between the multiport valve and the system outlet, and the extent of axial mixing, a cell-free cellulose bed was subjected to a square wave of FITC-insulin. Figure 3.2 shows the profile of FITC-insulin at the output in response to the square wave at the input. There was an approximately 4.5 min time lag for both step up and step down. This compares well

with the calculated residence time t=V/Q of 4 minutes, where V= 1 ml is the volume between the valve and the system outlet, and Q=0.25 ml/min is the perfusion flow rate. The more gradual ascent and descent of FITC insulin at the output relative to the input was apparently the result of some axial dispersion. The observed performance was adequate for evaluating the secretory characteristics of free and encapsulated cells with the accuracy needed to address the objectives of this study.

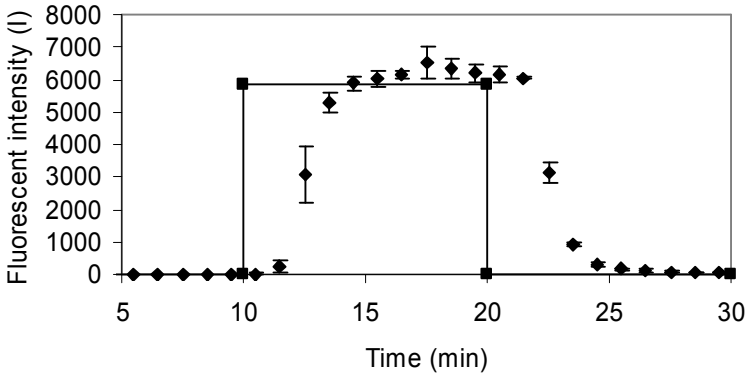


Figure 3.2 Profile of FITC-insulin at the output of perfusion system in response to a square wave at the input.

3.4.2 Secretion from islets

Figure 3.3 shows typical results from a perfusion experiment with porcine islets. Shortly after glucose stimulation, the insulin secretion rate reached its maximum. By 30 minutes post-stimulation, the insulin secretion rate had declined to approximately 50% of its highest value, and it continued to gradually decline for the rest of the induction period. One perfusion experiment was also performed with human islets (results not shown) and the secretion profile was similar to that shown in Fig. 3. This type of secretion profile is

similar to those reported with human islets in the literature [117].

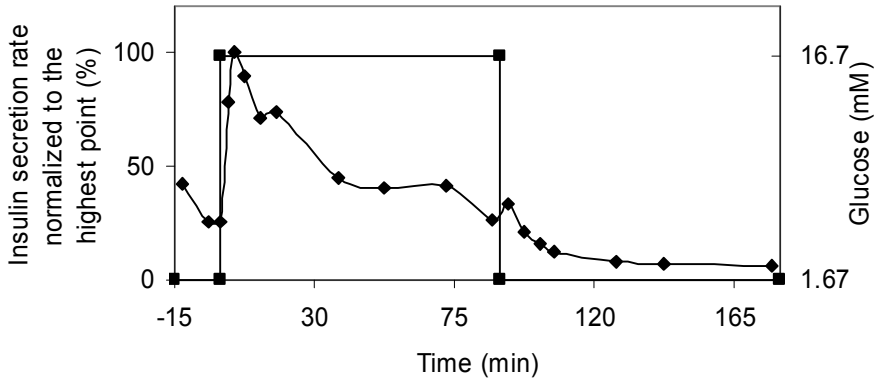


Figure 3.3 Dynamic secretory response of porcine islets subjected to a square wave of glucose concentration from 1.67 to 16.7 to 1.67 mM.

3.4.3 Effects of alginate composition on secretion dynamics of encapsulated βTC3 cells

βTC3 cells encapsulated in LVG and LVM exhibited different growth and remodeling patterns, as illustrated by the rate of glucose consumption (GCR) and the histological observation of beads at different time points (Figure 3.4). For LVM beads, cells overcame any growth inhibition quickly, and the GCR started to increase from day 4 onward. Histology slides indicated that remodeling inside the LVM alginate was diffusion driven: cells grew and formed clusters at the periphery of beads where oxygen was more available. For LVG beads, there was a prolonged growth suppression of cells, with GCR being essentially constant for 20 days before starting to increase. Cell growth resulted in clusters randomly dispersed throughout the beads. When normalized to GCR, the rate of insulin secretion (ISR) in the T-flask cultures was lower at the end of the

long-term cultures for both types of alginate. Specifically, the ISR/GCR ratio declined from 2.79±0.46 (mean ± std. dev.) μU/nmole on day 5 to 1.55±0.33 μU/nmole on day 19 for the LVM culture, and from 3.16±0.14 μU/nmole on day 2 to 0.72±0.26 μU/nmole on day 38 for the LVG culture. These trends are all consistent with trends reported by Stabler et al [114, 115].

The secretion dynamics of freshly trypsinized βTC3 cells loaded with cellulose in the perfusion chamber are shown in Figure 3.5a. Free cells responded essentially immediately to glucose stimulation, as indicated by the rapid increase in insulin secretion rate from the first sample taken after the system time lag. The insulin secretion rate decreased to below 50% of its highest value after 30 minutes of glucose stimulation. Secretory responses of LVG-encapsulated cells are shown in the other panels of Figure 3.5 and of LVM-encapsulated cells in Figure 3.6. The encapsulation process did not affect the glucose-stimulated secretion dynamics, as freshly encapsulated cells in both LVM and LVG exhibited secretion profiles very similar to those of free cells (day 2 LVG: Figure 3.5b; day 3 LVM: Figure 3.6a). Perfusion results from beads propagated for a period of time, in particular LVG-encapsulated cells on day 38 (Figure 3.5d) and LVM-encapsulated cells on day 20 (Figure 3.6c), indicated that capsules retained their fast response to glucose stimulation, but a high rate of secretion was maintained longer under glucose stimulation relative to the free and freshly encapsulated cells. Specifically, the insulin release rate was maintained above 50% of its highest value after 30 minutes of glucose stimulation. Results from intermediate time points (LVG day 20, Figure 3.5c, and LVM day 13, Figure 3.6b) differed depending on the type of alginate. With high M beads, the secretion profile was comparable to freshly encapsulated beads,

whereas with high G beads, the profile was comparable to that of the final time point. With both types of alginate and at all time points, insulin secretion returned to its basal rate within 15 minutes after the glucose step down.

The characteristics of the above secretion dynamics patterns are summarized in Table 3.1. The percentage of induced insulin secreted during the first 25 minutes was calculated by dividing the amount secreted during the first 25 minutes post-induction by the total amount secreted during the 1.5 hours of glucose stimulation. The glucose induction-fold reported in Table 3.1 was calculated by dividing the total amount of insulin secreted during the 1.5 hours of induction by the total amount of insulin secreted during the following 1.5 hours of exposure to glucose-free basal medium. The sharpness of the secretory profile of free and freshly encapsulated in both LVG and LVM βTC3 cells is reflected by the higher values of the percentage of induced insulin secreted during the initial 25 min after the glucose step up. Following long-term culture, the percentage of induced insulin secreted during first 25 minutes decreased, as the secretion profiles during high glucose became broader, and the induction-fold also decreased.

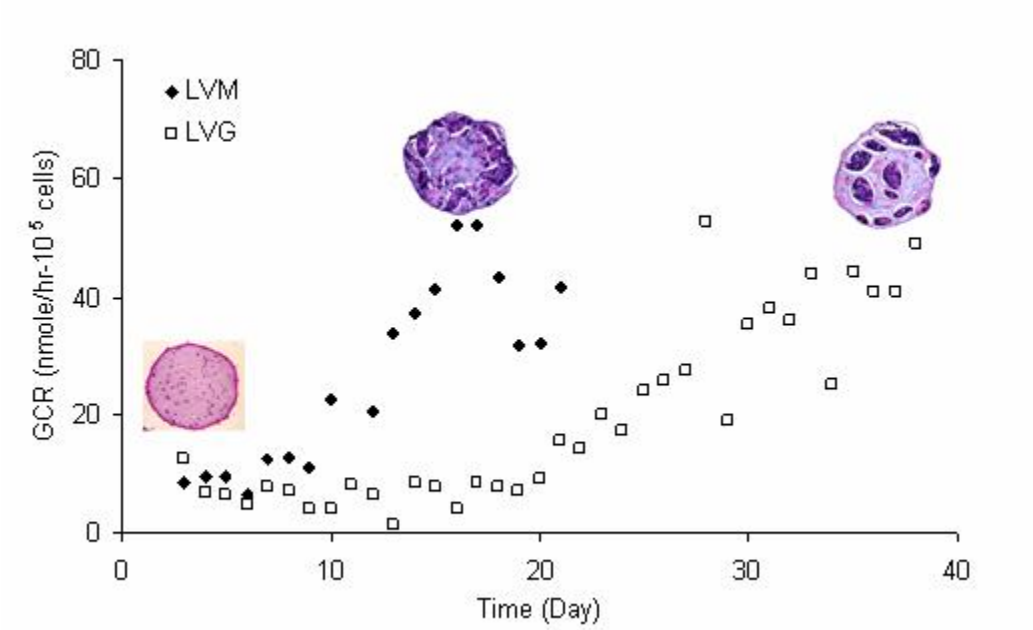
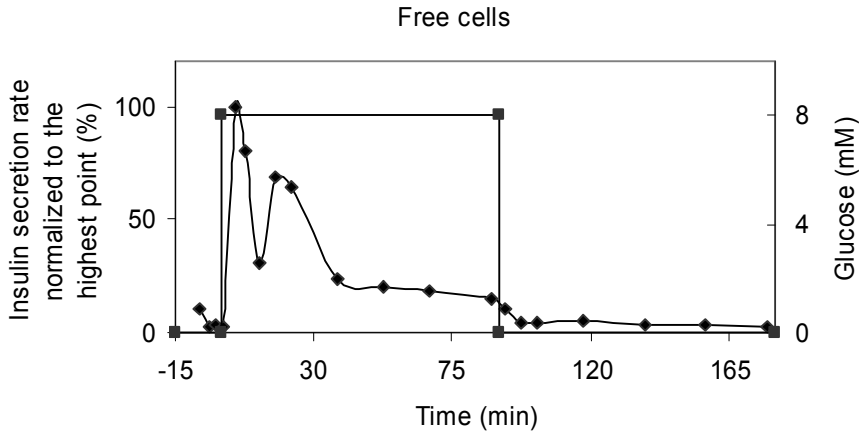


Figure 3.4 Temporal changes in the rates of metabolic activity (glucose consumption rate) for βTC3 cells encapsulated in 2% LVG (open squares) and 2% LVM (solid diamonds)-based APA beads. Representative histology cross-sections of APA-encapsulated βTC3 cells are: LVM beads at day 2 (left), LVM beads at day 20 (middle), and LVG beads at day 38 (right).

a.



b.

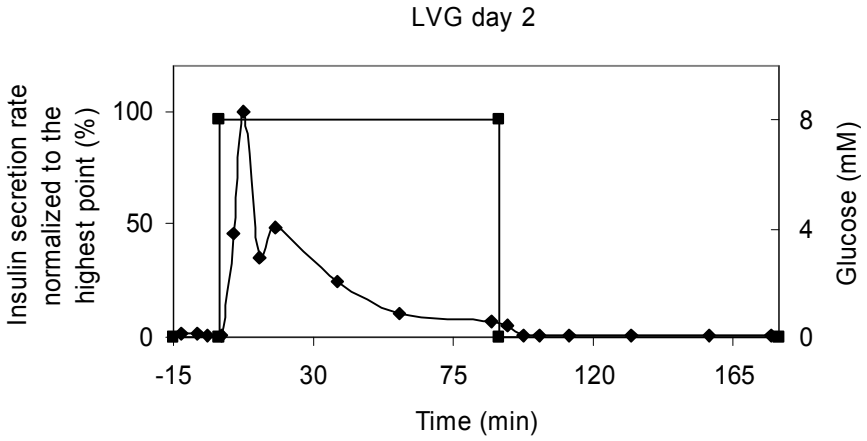
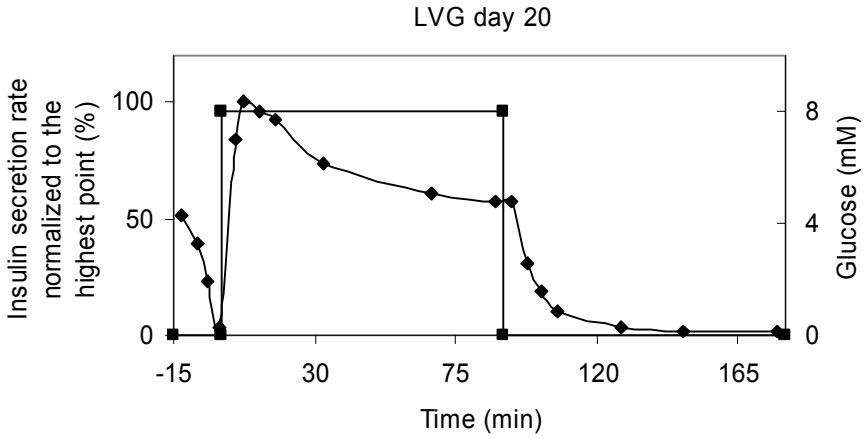


Figure 3.5 Dynamic secretory response of free βTC3 cells (a) and of 2% LVG-encapsulated βTC3 cells at day 2 (b), day 20 (c), and day 38 (d) subjected to a square wave of glucose concentration from 0 to 8 to 0 mM.

Figure 3.5 (continued).

c.



d.

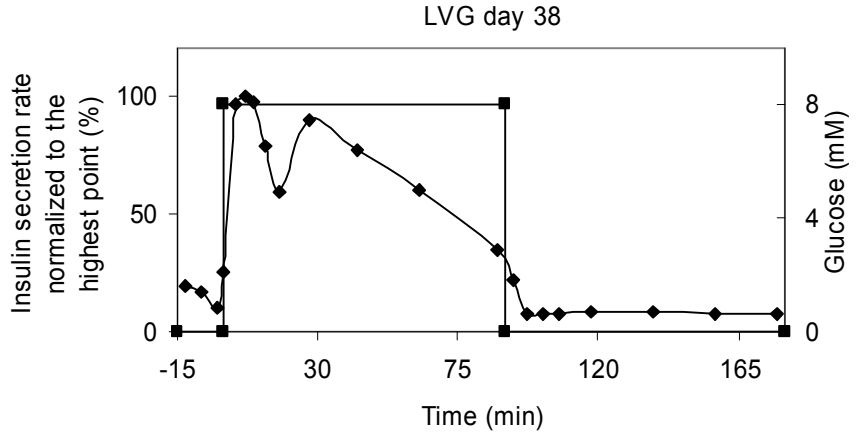
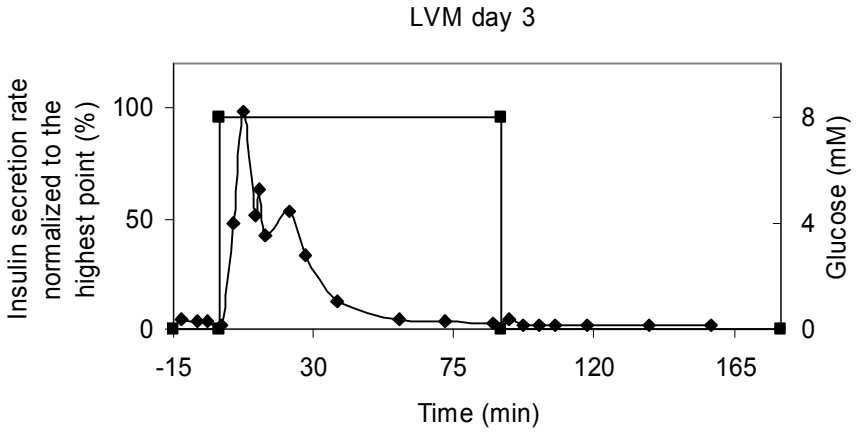


Figure 3.5 Dynamic secretory response of free βTC3 cells (a) and of 2% LVG-encapsulated βTC3 cells at day 2 (b), day 20 (c), and day 38 (d) subjected to a square wave of glucose concentration from 0 to 8 to 0 mM.

a.



b.

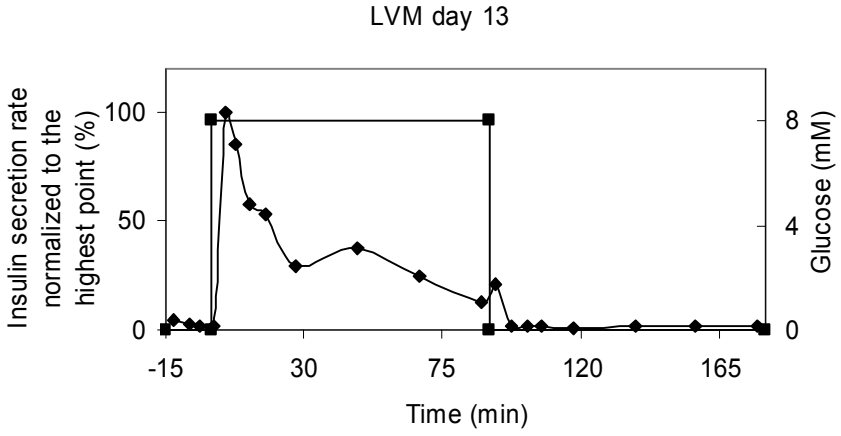


Figure 3.6 Dynamic secretory response of APA-encapsulated βTC3 cells in 2% LVM-based capsules at day 3 (a), day 13 (b), and day 20 (c) subjected to a square wave of glucose concentration from 0 to 8 to 0 mM.

Figure 3.6 (continued).

c.

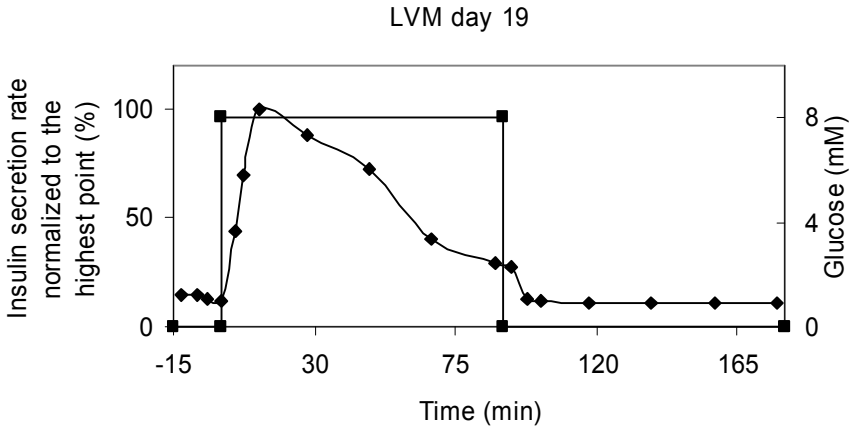


Figure 3.6 Dynamic secretory response of APA-encapsulated βTC3 cells in 2% LVM-based capsules at day 3 (a), day 13 (b), and day 20 (c) subjected to a square wave of glucose concentration from 0 to 8 to 0 mM.

Table 3.1 Summary of secretion characteristics of free and encapsulated βTC3 and βTC tet cells

	Free βTC 3 cells	**LVM day 2**	**LVM day 13**	**LVM day 20**	**Porcine islets**
% of insulin secreted during the first 25 minutes	33.50±3.41 (n=5)	38.67±7.23 (n=2)	39.16±12.00 (n=3)	24.00±10.14 (n=3)	49.13±6.03 (n=2)
Glucose induction-fold	5.87±1.58 (n=5)	11.18±0.95 (n=2)	8.36±2.36 (n=3)	4.68±1.68 (n=3)	5.74±0.62 (n=2)
		LVG day 2	**LVG day 20**	**LVG day 38**	
% of insulin secreted during the first 25 minutes		39.00±1.00 (n=3)	25.00±3.46 (n=3)	25.67±2.88 (n=3)	
Glucose induction-fold		11.68±8.59 (n=3)	6.30±3.99 (n=3)	3.83±0.95 (n=3)	
	Free βTC-tet cells	**Tet day2**	**Tet day20**	**Tet day30 (w/o tet)**	**Tet day30 (with tet)**
% of insulin secreted during the first 25 minutes	24.93±0.62 (n=3)	30.30±10.58 (n=3)	40.47±10.66 (n=3)	24.88±6.84 (n=3)	41.89±8.96 (n=3)
Glucose induction-fold	4.80±2.01 (n=3)	4.86±0.90 (n=3)	2.25±0.88 (n=3)	1.27±0.03 (n=3)	2.48±0.47 (n=3)

3.4.4 Effects of growth suppression on secretion dynamics of encapsulated βTC-tet Cells

Figure 3.7 shows the glucose consumption rate (GCR) and representative histology cross-sections of encapsulated βTC-tet cells as functions of time. Encapsulated cells were propagated in tetracycline-free medium for 20 days, at which point the culture was split in two: half the amount of beads continued to be propagated in the absence of tetracycline, whereas the other half was cultured in the presence of 30 ng/ml of the antibiotic. The βTC-tet cells in ISP alginate exhibited a long lag before GCR started to increase. In the absence of tetracycline, GCR started to increase around day 20, and by day 30 histology revealed a dense peripheral band of cells, indicating that the cell remodeling was also primarily driven by oxygen availability. Beads propagated with 30 ng/ml of tetracycline maintained the same glucose consumption rate, and histology at day 30 revealed a pattern similar to that observed with day 20 beads. These results are consistent with data from monolayer βTC-tet cell cultures which, when propagated with 30 ng/ml tetracycline after reaching 80% confluency, also stopped proliferating over a period of 10 days (results not shown). The time change of the (ISR/GCR) ratio of the encapsulated cells in the T-flask cultures depended on whether tetracycline was administered or not. In the absence of tetracycline, (ISR/GCR) declined from 5.8±1.76 μU/nmole on day 2 to 1.26±0.88 μU/nmole on day 30. On the other hand, when tetracycline was administered from day 20 to day 30, the (ISR/GCR) ratio on day 30 was 9.75±2.80 μU/nmole.

As with βTC3 cells, the encapsulation process had no immediate effect on the

secretion profile of βTC-tet cells. Beads cultured without tetracycline had a broader secretion profile at day 30 (Figure 3.8c) relative to day 20 beads (Figure 3.8a). On the other hand, the secretion profile from beads propagated with tetracycline from day 20 onward (Figure 3.8b) was very similar to the profile of day 20 beads, indicating that growth inhibition by tetracycline preserved the secretory profile. The spikes observed after the step down in glucose concentration were probably due to the sensitivity of βTC-tet cells to the small pressure change during medium switches. These spikes did not affect the overall secretory profile, including the sharpness of the response to the glucose step-up.

The secretion characteristics of βTC-tet cells are also listed in Table 3.1. Day 30 beads cultured without tetracycline had different secretion characteristics compared to day 20 beads, in that both the percentage of induced insulin secreted during the first 25 minutes and the induction-fold decreased. On the other hand, there were no statistical differences in these two parameters between day 20 and day 30 beads, when the latter were propagated with 30 ng/ml of tetracycline from day 20 onward.

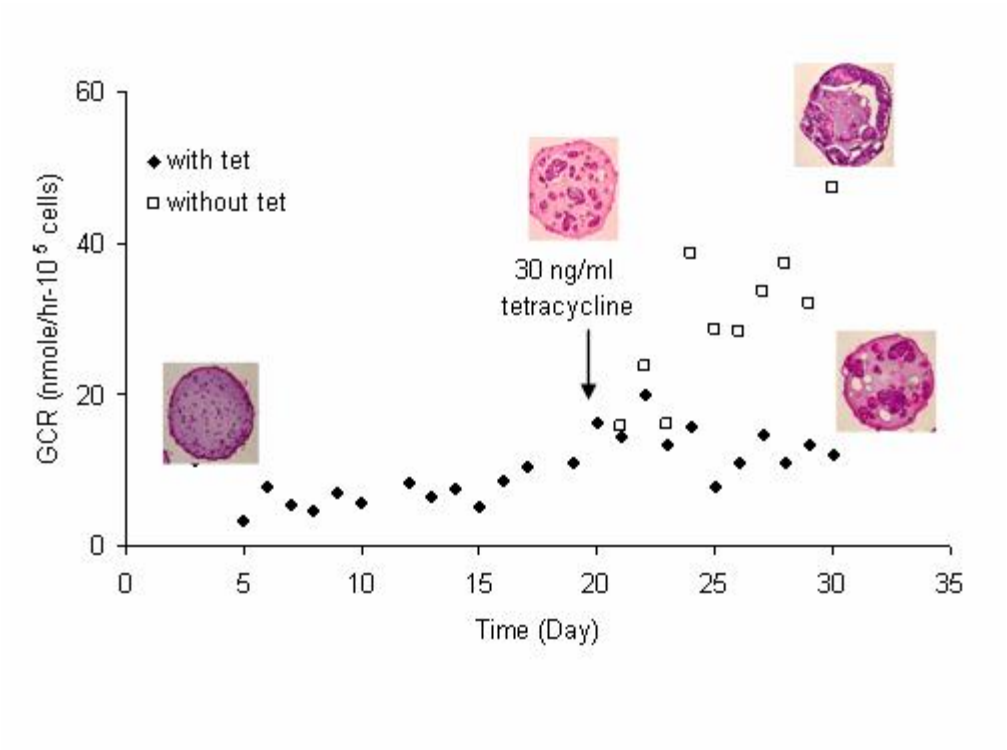
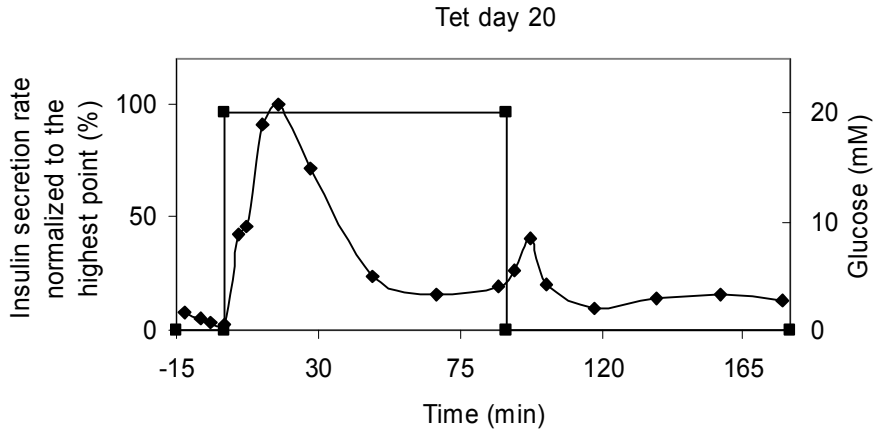


Figure 3.7 Temporal changes in the rates of metabolic activity (glucose consumption rate) for βTC-tet cells encapsulated in 2% alginate. Representative histology cross-sections of alginate encapsulated βTC-tet cells are (from left to right): day 2, day 20, and day 30 beads propagated with (bottom right) or without (top right) 30 ng/ml tetracycline.

a.



b.

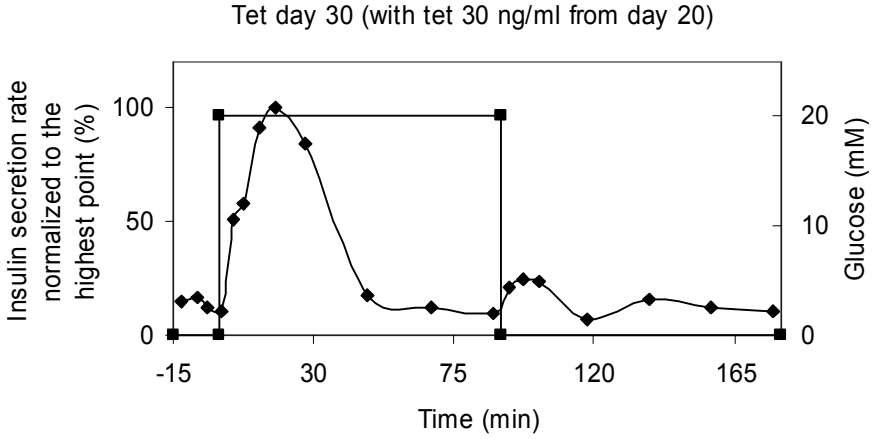


Figure 3.8 Dynamic secretory response of 2% APA-encapsulated βTC-tet cells at day 20 (a), and day 30 of beads propagated with (b) and without (c) 30 ng/ml tetracycline between day 20 and day 30 and subjected to a square wave of glucose concentration from 0 to 20 to 0 mM.

Figure 3.8 (continued).

c.

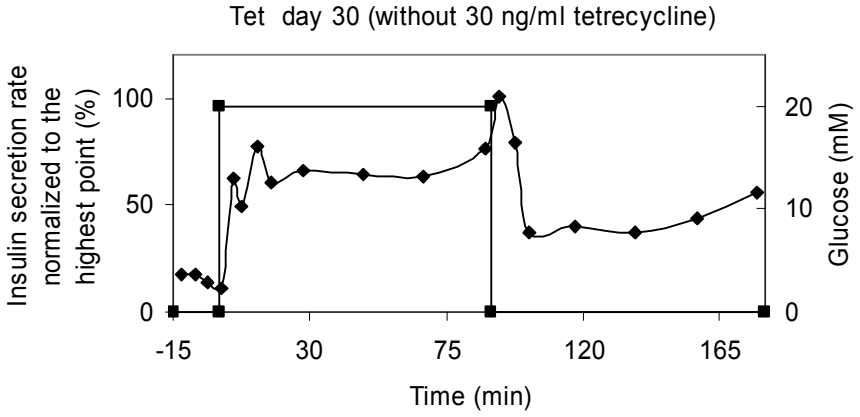


Figure 3.8 Dynamic secretory response of 2% APA-encapsulated βTC-tet cells at day 20 (a), and day 30 of beads propagated with (b) and without (c) 30 ng/ml tetracycline between day 20 and day 30 and subjected to a square wave of glucose concentration from 0 to 20 to 0 mM.

3.5 Discussion

In a cell-based therapy for insulin-dependent diabetes, the cells used should, ideally, exhibit the same insulin secretion dynamics as normal islets. Thus, the secretion of insulin should be glucose-dependent and exhibit fast kinetics in response to glucose and other physiologic stimuli. Static incubation of islets in media containing different glucose concentrations can elucidate the dependence of insulin secretion on glucose concentration. However, to capture the dynamic response of insulin release, a single pass perfusion system is more appropriate. Following a step up in glucose concentration, islets exhibit a biphasic insulin secretion profile. The initial acute phase of insulin

release is due to the discharge of insulin stored in readily releasable granules, whereas the second more prolonged phase of insulin secretion is attributed to elevated insulin biosynthesis and/or to the preparation of another group of granules for release [52, 53].

The biphasic insulin secretion pattern of islets has been reported to differ among different species [118]. Perfusion results with isolated mouse islets exhibited a sharp first phase of secretion, with the second phase of insulin release being at a flat and much lower rate than the maximum rate of the first secretion phase. In contrast, perfusion results with isolated rat islets indicated that the insulin secretion rate during the second phase was comparable to the maximum rate of the first phase. This difference has been attributed to different forms of cyclic AMP or the expression of distinct isoforms of protein kinase C in β cells of different species [119, 120]. However, it should be noted that differences in islet secretion profiles can be attributed not only to intrinsic biochemical differences but also to subtle differences in perfusion conditions. For instance, when isolated human islets were perfused *in vitro*, the insulin secretion profile was reportedly similar to the biphasic response of mouse islets [117, 121] and also resembled the secretion profile obtained in this study with porcine islets (Figure 3.3). On the other hand, when secretion from human islets was studied *in vivo* by the hyperglycemic clamp technique, in which the plasma glucose of a human test subject was subjected to a step increase and sustained at 10 mM for two hours by controlled glucose infusion, the secretion profile resembled that reported for rat islets [122, 123]. Free βTC3 and βTC-tet cells exhibited secretion profiles similar to those obtained porcine (Figure 3.3) and human islets in this study and reported *in vitro* with mouse and human islets elsewhere [117, 121]: an acute first phase of insulin secretion was followed by a

second lower and flat or slowly declining second phase of insulin secretion.

The similarity in secretion profiles of free and freshly encapsulated insulinomas indicated that the encapsulation process did not affect significantly the secretory cell function. Although the remodeling pattern was different for βTC3 cells in LVM and LVG APA beads, the changes in secretion profiles with time were similar for both types of alginate following recovery from growth suppression. After prolonged culture, the secretion profiles became broader, as indicated by the decrease of percentage of induced insulin secreted during the first 25 minutes after glucose stimulation, and the induction fold also decreased. Furthermore, when the insulin secretion rate was normalized to the glucose consumption rate, both measured in the T-flask cultures, the normalized insulin secretion rate declined with time in culture, possibly indicating a reduction in the overall secretory capacity of cells after prolonged propagation in APA beads.

There are several possible causes of the changes in insulin secretion profile exhibited by APA-encapsulated insulinomas with time in culture. One relates to changes in the cellular microenvironment. Shortly after encapsulation, cells were homogenously distributed as single units inside the beads. During the remodeling process, cells formed clusters and possibly deposited extracellular matrix [124], thus altering the cell-cell and cell-matrix interactions from those in freshly encapsulated preparations. Furthermore, the rates of transport of oxygen and other nutrients and metabolites inside the cell clusters are expected to be lower than the transport rates in the alginate gel [125], resulting in a broader distribution of the nutritive microenvironments experienced by cells after remodeling vs. initially. With the bead diameter and cell density used in this study, all encapsulated βTC3 cells were initially exposed to oxygen

concentrations above those limiting secretion [126]. However, after remodeling, it is likely that cells at the core of the clusters secreted insulin at reduced rates or not at all due to severe hypoxia at these locales. Identifying the exact cause of the secretory profile changes with time would be interesting and significant, but such an investigation was clearly beyond the scope of this study.

Previous studies [116] clearly indicate that encapsulated proliferative cells do not exhibit unlimited growth in capsules. Instead, there is growth to the number of viable cells supported by the oxygen concentration in the environment. At this point, the number of viable cells is maintained constant through equilibration of the rates of cell growth and death. Thus, growth arrest of the type that can be implemented with βTC-tet cells may not be necessary for providing an upper limit to the rate of insulin secretion exhibited by encapsulated cells; rather, it appears necessary for decelerating or arresting remodeling and inhibiting accumulation of dead cells in capsules, possibly also for inhibiting the growth of any cells released from broken capsules. As found in this study, the secretion profile of APA-encapsulated βTC-tet propagated in the absence of tetracycline, in which cells continued to grow and remodel, became broader from day 20 to day 30. On the other hand, with beads propagated in the presence of tetracycline, the secretion profile was preserved and the induction-fold was maintained at the same value. Thus, growth inhibition appears necessary for preserving the insulin secretion profile exhibited by an encapsulated cell system. Moreover, as indicated by the increase of the ISR/GCR ratio in encapsulated βTC-tet cells cultured in the presence of tetracycline, which in accord with previous findings with βTC-tet monolayers [127]: growth arrest may stimulate expression of the insulin gene and of genes encoding components of

insulin secretory vesicles. It should be noted that for encapsulated βTC-tet cells, both the concentration of tetracycline and the time of administration are critical in achieving growth arrest [128]. Thus, the in vivo implementation of this growth control mechanism may be challenging.

It remains unclear whether the changes in insulin secretion dynamics exhibited by proliferating encapsulated insulinomas would change their effectiveness in providing *in vivo* glycemic control. Such differences in effectiveness would be better manifested in large animal models, which have more elaborate glucose homeostasis relative to rodents. Besides characterizing the dynamic secretory changes occurring with time, this study clearly demonstrated that the method to maintain the secretory profile constant is growth suppression of the cells. Growth arrest, either through regulation of expression of the SV40 T antigen (Tag) oncoprotein by tetracycline, as in the βTC-tet cells, or through some other mechanism, could potentially be applied *in vivo* if changes in secretory profile prove to be detrimental for glycemic regulation.

CHAPTER 4

HYBRID PANCREATIC TISSUE SUBSTITUTE CONSISTING OF RECOMBINANT INSULIN-SECRETING CELLS AND GLUCOSE-RESPONSIVE MATERIAL

4.1 Abstract

Insulin-dependent diabetes is a serious pathological condition, currently treated by blood glucose monitoring and daily insulin injections, which, however, do not prevent long-term complications. A tissue engineered pancreatic substitute has the potential to provide a more physiologic, less invasive, and potentially less costly treatment of the disease. A major issue in developing such a substitute is the cells being used. Non-pancreatic cells, retrieved from the same patient and genetically engineered to secrete insulin constitutively or with some glucose responsiveness, offer the significant advantages of being immune acceptable and relaxing the tissue availability limitations which exist with islets from cadaveric donors. These cells, however, do not exhibit insulin secretion dynamics appropriate for restoration of euglycemia in higher animals and, eventually, humans. In this study, we present the concept of a hybrid pancreatic substitute consisting of such cells sequestered in a material exhibiting glucose-dependent changes of its permeability to insulin. A concanavalin A-glycogen material sandwiched between two polycarbonate membranes and exhibiting glucose-dependent sol-gel transformations was used. Rates of insulin transport through this material in gel and sol forms were characterized for both FITC-labeled insulin in solution and insulin secreted by βTC3 mouse insulinoma cells. Effective diffusivities through sol were found to be up to 3.5-fold higher than through the gel state of the material. A mathematical model

of a hybrid construct was formulated and analyzed to simulate the secretory behavior in response to step ups and downs in the surrounding glucose concentration. The experimental and modeling studies indicate that a hybrid pancreatic substitute consisting of constitutively secreting cells and glucose-responsive material has the potential to provide a more physiologic regulation of insulin release than the cells by themselves or in an inert material.

4.2 Introduction

Insulin dependent diabetes (IDD) is a serious pathological condition affecting more than 4 million people in the United States with a total estimated annual cost of more than $80 billion. The current treatment for this disease involves daily monitoring of blood glucose levels and multiple daily insulin injections. This provides some control of the diabetic state, which, however, is far from the physiologic regulation achieved by a normally functioning pancreas and results in long-term complications, including cardiovascular disease, nephropathy, cataracts, retinopathy and skin ulcers. A tissue-engineered pancreatic substitute consisting of insulin-secreting cells and biomaterials in a functional three-dimensional architecture has the potential to provide a more physiologic, less invasive, and potentially less costly treatment of the disease relative to insulin injections.

A major issue in developing a pancreatic substitute is the cells being used. A commonly studied configuration consists of pancreatic islets encapsulated in a permselective barrier for protection from the immune system of the host. However, survival and function of these implants does not occur reproducibly and for a sufficiently

long period to encourage human clinical studies. One possible cause of failure is that encapsulation provides only partial immune protection, since antigens released by implanted cells can pass through the membrane and cause immune activation of the host.

Autologous cells constitute a promising alternative to pancreatic islets, since they are accepted immunologically. Clearly, such cells have to be other than β pancreatic cells, since the latter have been destroyed or are seriously malfunctioning in insulin-dependent diabetics. Genetic engineering of non-β cells for constitutive expression of recombinant insulin is straightforward, and indeed several investigators have expressed insulin in fibroblasts, myoblasts, and hepatocytes, among other cell types [129-131]. Glucose responsiveness may be introduced at the gene level by using promoters up-regulated by glucose and possibly down-regulated by insulin to drive proinsulin cDNA transcription [83, 84, 132]. However, such cells cannot exhibit the acute dynamics of insulin secretion, which are needed for normoglycemia in higher animals and, eventually, humans [133]. To address this problem, we hypothesized that, by sequestering cells in a material exhibiting glucose-dependent permeability changes with a short time-constant, one could physically re-create a glucose-responsive insulin storage compartment, which the cells are lacking biologically. One such material is based on the lectin concanavalin A (con A) bound to the polysaccharide glycogen, which exhibits sol-gel transformations depending on the glucose concentration in the surrounding medium [134, 135]. This type of pancreatic substitute based on sub-optimally secreting cells sequestered in a glucose-responsive material constitutes a substantially different design from the encapsulated cell systems reported thus far, and poses a unique set of problems that need to be addressed prior to *in vivo* applications.

In this paper, we present this novel concept of the hybrid substitute consisting of cells and glucose-responsive material; we characterize the con A-glycogen complex as a glucose-sensitive barrier to insulin release from a solution or from a suspension of βTC3 mouse insulinoma cells; and, to illustrate the insulin secretion profile that can be exhibited by this device, we develop and analyze a mathematical model of a hybrid device subjected to cycles of glucose concentration similar to those occurring physiologically. The potential of this type of device for IDD treatment and areas where improvements are needed prior to *in vivo* studies are discussed.

4.3 Materials and Methods

4.3.1 Cells and cell culture

βTC3 cells were obtaind from the laboratory of Shimon Efrat, Albert Einstein College of Medicine, Bronx, NY. Cells of passage number 42-45 were used in this study. Cells were cultured as monolayers in T-flasks with culture medium changed every 2-3 days. Culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis MO) with 25 mM glucose, supplemented with 15% horse serum (Sigma), 2.5% bovine serum (Mediatech, Herndon, VA), 1% penicillin-streptomycin (Mediatech) and L-glutamine (Gibco, Grand Island, NY) to a final concentration of 6 mM. Upon reaching 100% confluence, cells were harvested by trypsin-EDTA (Sigma) and encapsulated in 2% Keltone alginate (ISP Alginates Inc., San Diego, CA) at a density of $3x10^7$ cells/ml alginate using an electrostatic droplet generator (Nisco Engineering Inc., Zurich, Switzerland) and following the procedure of Stabler et al. [71], except that the poly-L-lysine and final alginate layers were not added. The final

bead size was 1 mm in diameter.

4.3.2 Glucose-responsive material

The con A-based material was prepared followed the general procedure of Tanna et al. [134, 136]. In initial experiments with dextran, the con A-dextran material proved to be less sensitive to glucose than the material with glycogen, hence glycogen was used in this study. Glycogen (type IX, G-0885), Concanavalin A (type V, C-7275), sodium m-periodate ($NaIO_4$), and sodium borohydride ($NaBH_4$) were all from Sigma. In short, glycogen was first dissolved in PBS (pH=7.2) to form a concentrated solution of 60% w/v. To activate the glycogen to the aldehydic form, $NaIO_4$ was added and mixed with glycogen in the dark for 24 hr to final concentrations of 0.1 M and 30% w/v for $NaIO_4$ and glycogen, respectively. Following this, concanavalin A dissolved in PBS at 15% w/v was added to the activated glycogen at a 1:1 volume ratio. Stirring of the mixture started immediately and continued until a viscous gel was formed. The gel was left at room temperature for 24 hours, then a 1 mg/ml of $NaBH_4$ solution was added at a 2:1 volume ratio, and the material was immediately placed on a shaker at 4^{o}C for 1 hr to stabilize the gel. Finally, the gel was rinsed with PBS for at least 3 times and stored at 4^{o}C before use. To test the phase changes of the material in different glucose concentrations, an amount of the material in PBS solution was loaded in a dialysis tube (12,000 MWCO, Sigma) and immersed in PBS, either glucose-free or containing glucose at 4% concentration.

4.3.3 Diffusion experiments and assays

To measure the rate of insulin transport through the con A-glycogen material in the presence or absence of glucose, the two-compartment diffusion apparatus shown in

Figure 4.1 was used. The compartments were separated by the con A-glycogen material sandwiched between two 0.1 μm pore size polycarbonate membranes (Osmonics Inc., Minnetonka, MN) positioned 2 mm apart by a silicon sheet. The solutions in the donor and receiver compartments were mixed with a mixer or a stir bar to avoid boundary layer effects. To load the material homogeneously, 0.5 ml of material was first transformed into sol by the addition of an equal volume of 10% glucose solution. Following assembly of the entire diffusion apparatus, the material was injected into the space between the two membranes. The sol was converted to gel in glucose-free PBS solution for 20 minutes prior to the beginning of diffusion experiments.

For experiments with insulin solutions, 1 mg/ml FITC-insulin (Sigma) in PBS was added to the donor compartment, whereas the receiver compartment was loaded with initially insulin-free PBS. To convert a gel to sol, glucose was added to a final concentration of 4% in both compartments. To convert a sol to gel, the solutions in both compartments were changed to glucose-free PBS. Samples were taken every 15 minutes, and FITC-insulin concentrations were measured by a fluorescence plate reader (Spectra Max Gemini Plate Reader, Molecular Devices Corp., Sunnyvale, CA) using a standard curve constructed on the basis of known FITC-insulin concentrations. Experiments with FITC-insulin were performed at room temperature.

To measure the amount of con A leakage during a gel-sol-gel cycle, the set up described above was used except that insulin-free PBS was loaded in both compartments. Samples from both compartments were assayed for total protein concentration by Coommassie Blue (Pierce, Rockford, IL). The amount of leakage was calculated based on assumption of no loss of con A during material preparations.

In experiments with encapsulated βTC3 cells as the insulin source, 1.5 ml of alginate beads with cells were incubated in a T-25 flask in 5 ml of culture medium with 20 mM glucose for 3 hours; during this time, secreted insulin accumulated in the medium. Beads and medium were then transferred to the donor compartment of the diffusion apparatus. Fresh culture medium with 20 mM glucose was added to the receiver compartment and the entire apparatus was placed in a 37^{o}C, 5%CO_2 humidified incubator. Samples were taken every 30 minutes and frozen until assayed. Insulin concentrations were measured by a double-antibody rat radioimmunoassay kit (Linco Research, St. Charles, MO), which exhibits a 100% cross-reactivity against mouse insulin (manufacturer's data). Cell viabilities were measured by dissolving the beads in 2.2% sodium citrate. Cells were pelleted by centrifugation at 105 g for 3 min; pellets were re-suspended in fresh medium and cell viability was measured by adding trypan blue (Sigma).

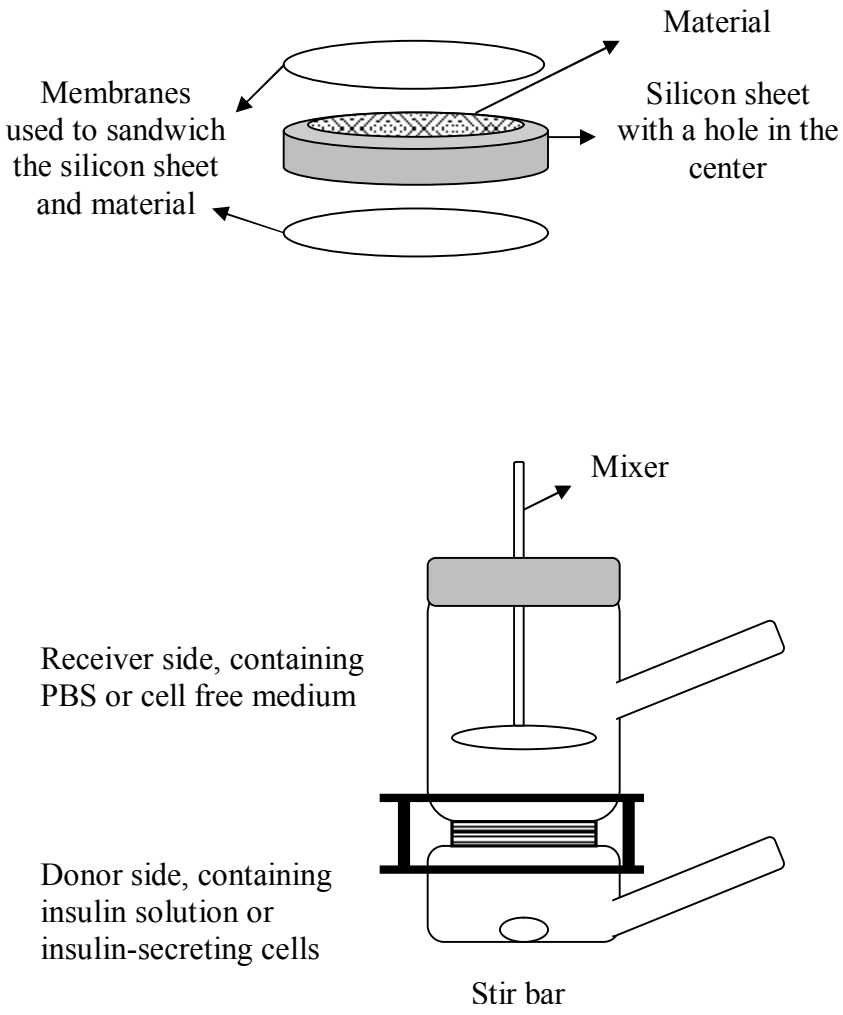


Figure 4.1 Diffusion apparatus for measuring insulin transport rates through the con A-glycogen material in gel and sol states. The material was sandwiched between two 0.1 μm pore size polycarbonate membranes positioned 2 mm apart by silicon sheeting with a 2 cm diameter hole in the middle. During preparation, the material was injected in sol state through the silicon sheeting to fill the space between the two membranes. The donor compartment, containing an insulin solution or insulin-secreting cells, had a volume of 6.5 mL. The receiver compartment, in which the concentration of insulin transported through the material was measured, had a volume of 10 mL. The area through which mass transport occurred was 3.14 cm^2

4.4 Results

4.4.1 Experimental studies

The chemical structure of the con A-glycogen material is illustrated schematically in Figure 2.10 and the macroscopic appearance of the gel and sol states in Figure 4.2. In the latter figure, the material is in a dialysis tube of 12,000 molecular cut off to prevent leakage of con A and glycogen. In glucose-free PBS solution, con A forms a three-dimensional gel matrix with glycogen. When the glucose concentration is increased, glucose displaces con A from the matrix, the three-dimensional structure breaks down, and the gel becomes a sol. When glucose is removed, the gel structure reforms. We have been able to repeatedly transform the material in a dialysis tube from the gel to the sol state and vice versa by changing the glucose concentration between 0% and 4% (220 mM).

The rates of insulin transport through the gel and sol states of the material were studied with the two-compartment apparatus illustrated in Figure 4.1. Figures 4.3 and 4.4 show results from typical experiments at room temperature with FITC-labeled insulin loaded in the donor compartment and assayed by fluorescent spectroscopy in the receiver compartment. In the experiment of Figure 4.3, two diffusion chambers were run in parallel. In apparatus 1, mass transfer of FITC-insulin was studied for 180 min with the material being always in the sol state. In apparatus 2, the material was initially in the gel state; after 180 min, glucose was added to both compartments to a concentration of 220 mM, converting the material to sol. At 360 min, the solutions in both compartments were replaced with fresh glucose-free solutions; the solution in the receiver compartment was insulin-free and the solution in the donor compartment contained FITC-insulin.

Accumulation of FITC-insulin in the receiver compartment was in all cases measured as a function of time.

Effective diffusivities D_{eff} were calculated from the linear portions of the concentration profiles using equation (1) below and are reported in Figure 4.3.

$$\ln\{[\alpha C_{A0} + C_{B0} - C_B(1+\alpha)]/[\alpha(C_{A0} - C_{B0})]\} = -[(1+\alpha)/\alpha](AD_{eff}/LV_B)t \quad (1)$$

(from [137])

where V_A=volume of donor compartment; V_B=volume of receiver compartment; $\alpha = V_A/V_B$; C_A=insulin concentration in donor compartment; C_B=insulin concentration in receiver compartment; C_{A0}, $C_{B0}=C_A$, C_B, respectively, at time 0; L=thickness of material separating the two compartments; A=area through which insulin transport occurs; D_{eff}=effective diffusivity. The slopes of the concentration profiles indicate that the gel to sol transformation was completed in less than 40 min, whereas the sol to gel transformation occurred much slower and was not completed by the end of the experiment.

Figure 4.4 shows results from a similar experiment, in which the sequence of material transformations in one of the apparati was in reverse order to that of Figure 4.3. In apparatus 1, mass transfer of FITC-insulin was studied for 180 min with the material being in the gel state. In apparatus 2, the material was initially in the sol state; after 180 min, the solutions in both compartments were replaced with fresh glucose-free solutions, with the solution in the receiver compartment being insulin-free and the solution in the donor compartment containing FITC-insulin. At 360 min, glucose was added to both compartments of apparatus 2 to a concentration of 220 mM. The accumulation of FITC-insulin in the receiver compartment was in all cases measured as a function of time, and effective diffusivities D_{eff} were calculated from equation (1) above and are reported

in the figure.

The average effective diffusivities of FITC-insulin, calculated for the material in the gel and sol states, are presented in Figure 4.5. Data are expressed as the mean ± standard deviation. Statistical differences were evaluated by the Student's t-test. Differences were considered significant at $p<0.05$.

To assess the extent of material leakage through the polycarbonate membranes, following the experiments in Figures 4.3 and 4.4, the solutions in both chambers were changed to glucose-free PBS and the apparati were incubated overnight. Some leakage of macromolecules was detected especially for the sol to gel to sol apparatus (Figure 4.4): the material recovered following the overnight incubation was of approximately 0.4 ml volume, whereas the material initially sandwiched between the two membranes had a volume of 0.5 ml. Additionally, the concentration of leaked con A was measured by Coommassie Blue in an insulin-free apparatus. It was found that approximately 10% of the initial con A amount leaked from the membrane sandwich by the end of a gel-sol-gel cycle.

To illustrate that the glucose-responsive material can be combined with cells to create a hybrid device with improved secretion properties, the same diffusion apparatus was used, except that in this case the donor compartment contained mouse βTC3 insulinomas. To keep the cells in suspension without compromising their viability and functionality, the cells were encapsulated in 1 mm diameter, 2% calcium alginate beads at a density of $3x10^7$ cells/ml alginate. The diffusion apparatus was placed in a 37°C, 5%CO_2 humidified incubator. Insulin secreted from βTC3 cells had accumulated in the donor compartment to approximately 150 ng/mL by the beginning of an experiment, and

it reached approximately 200 ng/mL by the experiment end. These concentrations are much higher than those in the receiver compartment, which did not exceed 10 ng/mL. It should be noted that unless the glucose concentration drops well below 1 mM, TC3 cells are constitutive secretors of insulin [5]. This is because these cells overexpress the low K_m glucose transporter GLUT1, as opposed to GLUT2, and hexokinase, as opposed to glucokinase, and consequently are hypersensitive to glucose [5, 68]. Thus, in the experiments of this study, the βTC3 cells were constitutive secretors of insulin.

Figure 4.6 shows results from a typical experiment, in which the donor compartment contained alginate-entrapped βTC3 insulinomas, the receiver compartment was initially insulin-free, and the material was in the gel state. At 180 min, glucose was added to the both compartments at a concentration of 220 mM transforming the material to sol. Insulin that had accumulated in the donor compartment while the material was in the gel state was released when the material was converted to sol. Calculated effective diffusivities are indicated in Figure 4.6.

Figure 4.7 shows insulin effective diffusivities of the material from experiments with the diffusion apparatus containing βTC3 cells in the donor compartment. Data were obtained with the material in the gel state for the duration of the experiment (bar A), the material in the gel state for the first 180 min (bar B) then converted to sol, and the material in the sol state following addition of 220 mM glucose at 180 min (bar C). Data are expressed as mean ± standard deviation. The higher effective diffusivities measured with βTC3 cells (Figure 4.7) relative to FITC-insulin (Figure 4.5) are probably due to the different conditions in the two sets of experiments, including temperature (37^{o}C vs. room temperature), solution (culture medium vs. PBS), and structure of diffusing molecule

(secreted insulin vs. FITC-insulin).

Overnight incubation of the apparatus in glucose-free PBS following the end of experiments with cells in the donor compartment indicated similar leakage of macromolecules as in the cell-free diffusion experiments (Figures 4.3 and 4.4). To further assess the toxicity of leaked con A, alginate-entrapped βTC3 cells were incubated in the donor compartment of the diffusion apparatus with con A-glycogen sandwiched between polycarbonate membranes under the following conditions: DMEM with 20 mM glucose (material in gel state) for 6 hours; DMEM with 220 mM glucose (material in sol state) for 6 hours; DMEM with 20 mM glucose for 3 hours followed by DMEM with 220 mM glucose for an additional 3 hours (material initially in gel state then converted to sol). Cell viability was measured by trypan blue at the end of each test. Results are shown in Table 4.1. No significant decrease in cell viability was observed at the end of each test.

Table 4.1 Viabilities of alginate-entrapped βTC3 cells incubated in the donor compartment of the diffusion apparatus for 6 hours with material in sol or gel state, or initially gel converted to sol 3 hours in the experiment

	sol	gel	gel to sol
Viability (%) at the end of each 6 hr test	87 (n=2)	90 (n=3)	87.6 (n=3)

Gel Sol

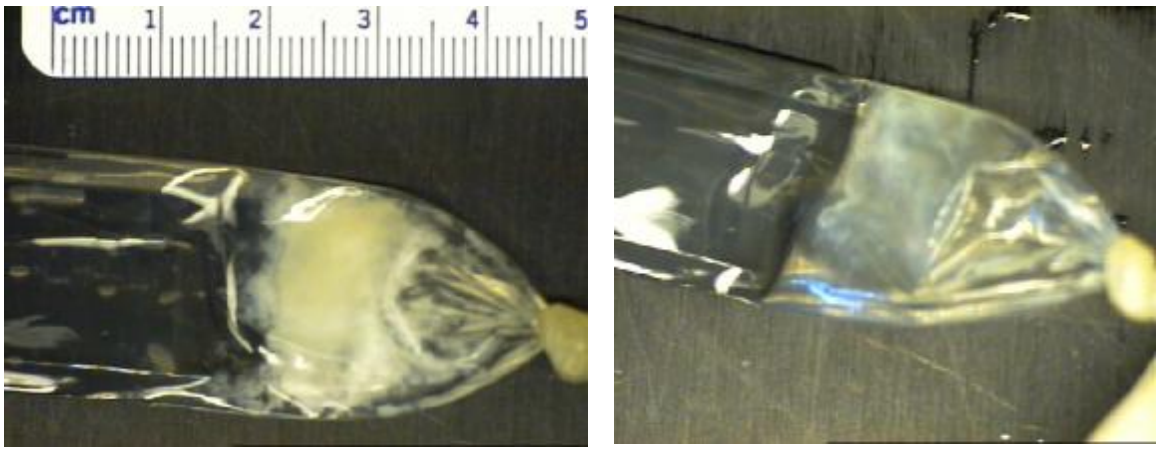


Figure 4.2 Macroscopic appearance of Concanavalin A-glycogen material exhibiting sol-gel transformations according to the glucose concentration in the surrounding medium in a dialysis tube.

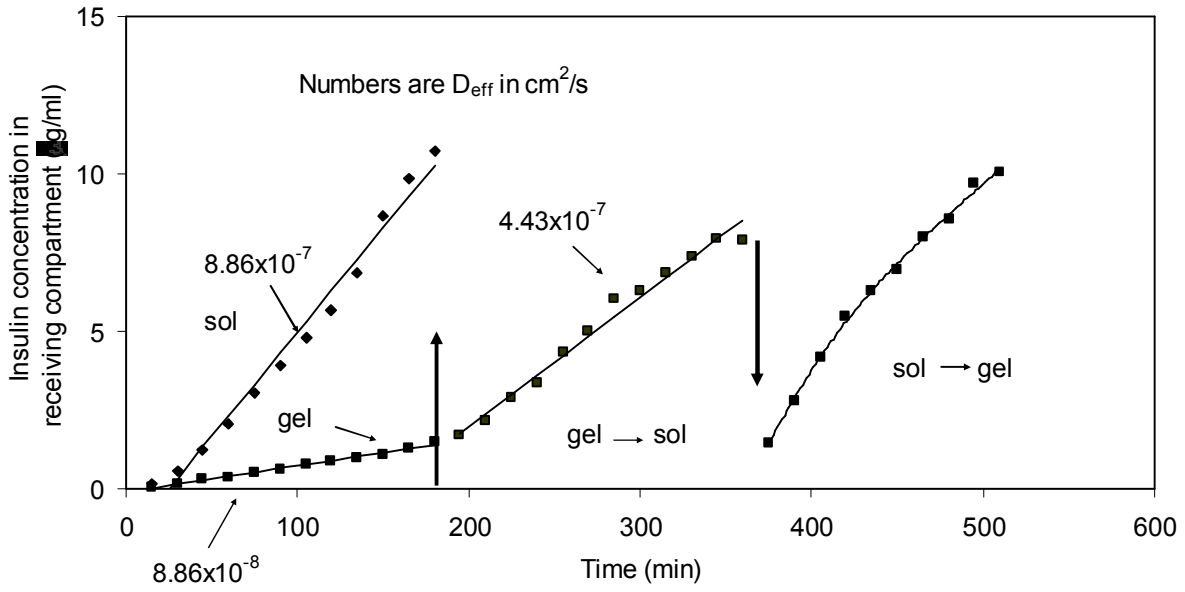


Figure 4.3 Accumulation of FITC-insulin in the receiver compartments of two diffusion apparati containing FITC-insulin in the donor compartments. Diamonds: apparatus 1; squares: apparatus 2. Initially, both receiver compartments were insulin-free. Straight lines are least squares fits of the data; curved lines are polynomial fits to the data.

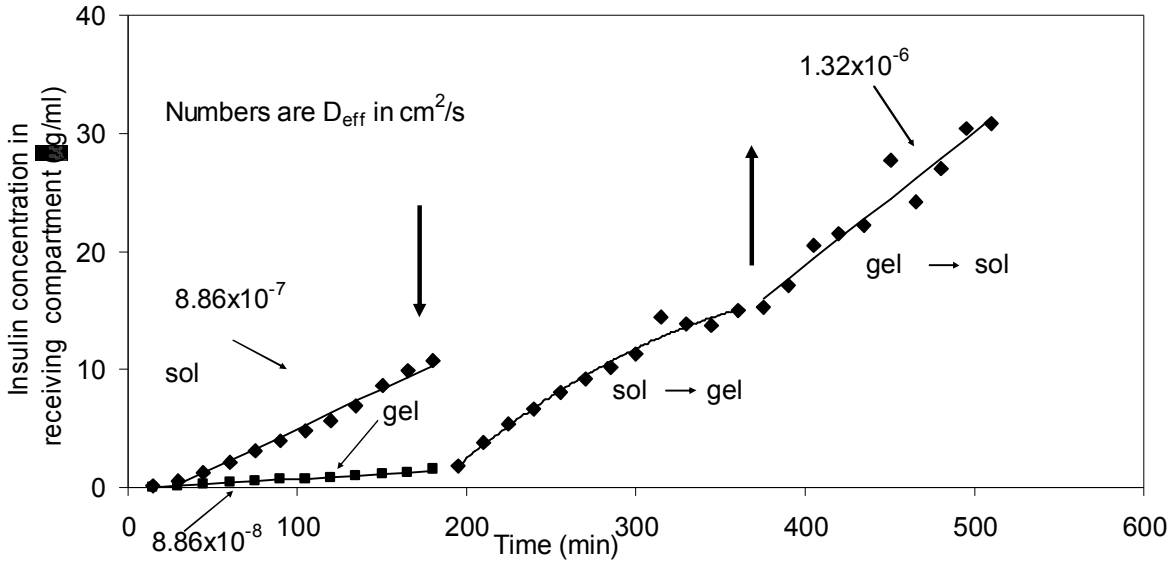


Figure 4.4 Accumulation of FITC-insulin in the receiver compartments of two diffusion apparati containing FITC-insulin in the donor compartments. Squares: apparatus 1; diamonds: apparatus 2. Initially, both receiver compartments were insulin-free. Straight lines are least squares fits of the data; curved lines are polynomial fits to the data.

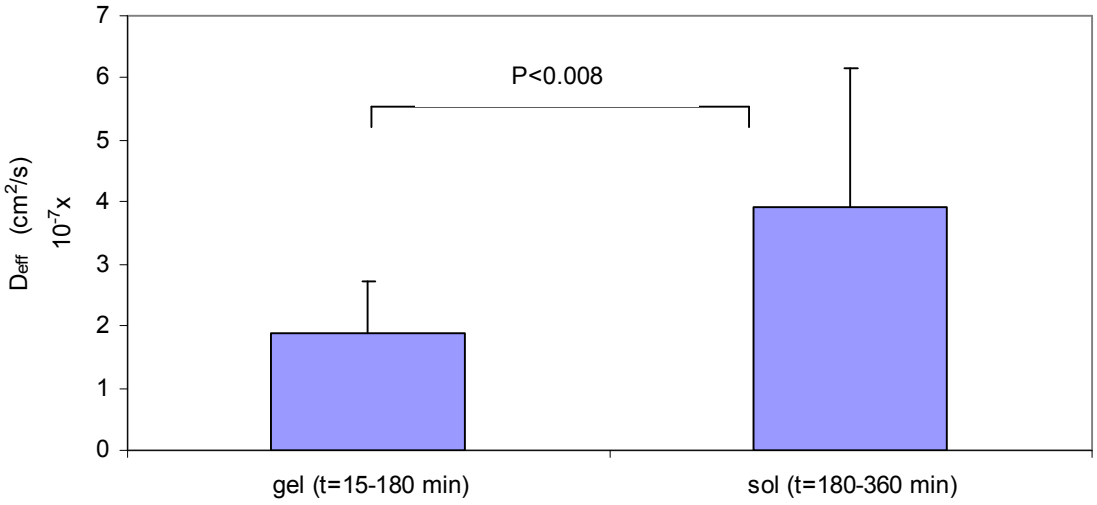


Figure 4.5 Average effective diffusivities of FITC-insulin, calculated for the material in the gel and sol states, from a total of 8 experiments of the type shown in Figure 4.3 with apparatus 2 during the initial 360 min. Glucose-free solutions were used in the diffusion apparatus for 180 min, after which glucose was added to both compartments to a concentration of 220 mM.

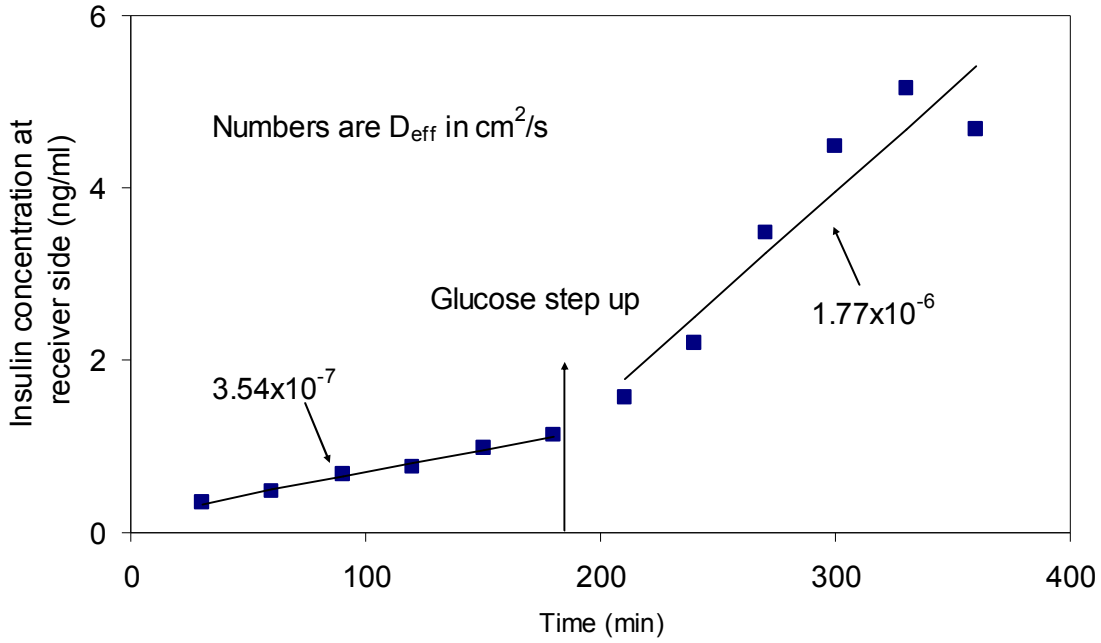


Figure 4.6 Accumulation of insulin in the receiver compartment of a diffusion apparatus containing alginate-encapsulated mouse βTC3 insulinomas in the donor compartment. Initially, the material was in the gel state and the receiver compartment was insulin-free. At 180 min, glucose was added to both compartments to a concentration of 220 mM causing the material to change to sol. Straight lines are least squares fits of the data.

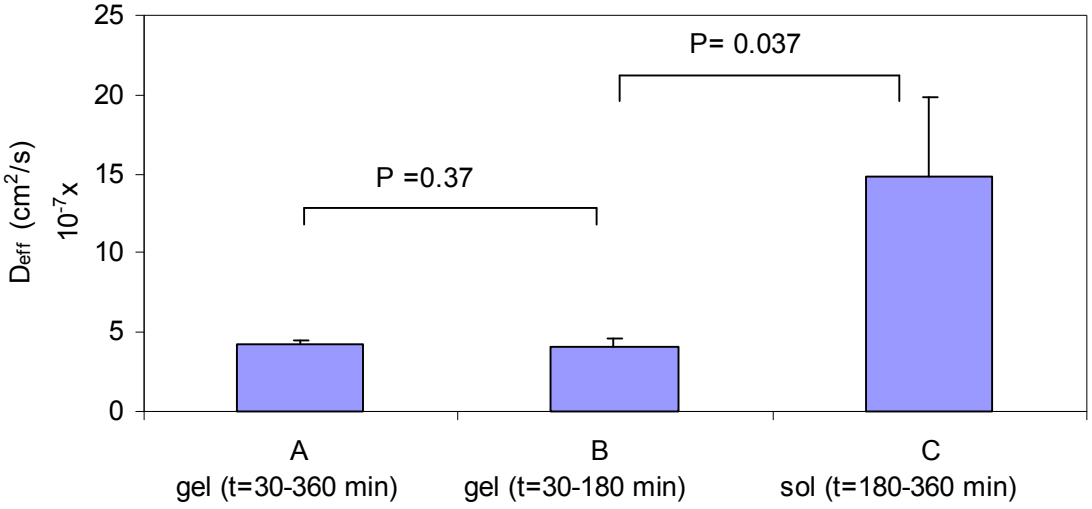


Figure 4.7 Insulin effective diffusivities of the material from experiments with the diffusion apparatus containing βTC3 cells in the donor compartment. Bar A corresponds to diffusivities calculated with the material always in the gel state during the experiment. The data for bars B and C were calculated from experiments of the type shown in Fig. 6. Bar B corresponds to diffusivities calculated with the material in gel state during the first 180 min of the experiment; after this, the material was converted to sol, and diffusivities with the material in the sol state are shown by bar C. For all bars, n=3.

4.4.2 Mathematical modeling[1]

To provide proof-of-concept that a hybrid construct based on constitutively secreting cells and on glucose-responsive material can release insulin with improved dynamics for physiologic glucose regulation, a mathematical model was developed to simulate the behavior of such a device. The architecture considered is illustrated in Figure 4.8. Cells dispersed in a gel are enclosed in a flat, cylindrical construct and are surrounded by a combination of the glucose-responsive material and a material permeable to oxygen and impermeable to insulin, such as silicone. The glucose-responsive material, sandwiched between two membranes as before, occupies one of the flat surfaces of the apparatus. The cylindrical and the second flat surfaces are occupied by the silicone-type material. Thus, insulin diffusion occurs only through one of the flat surfaces, whereas oxygen diffusion can take place via both the flat and cylindrical surfaces of the device.

The device is scaled for the diabetic mouse as a small animal model. Previous work with βTC3 cells has shown that $3x10^6$ cells are needed to restore normoglycemia in NOD mice (C. Weber and S. Safley, personal communication). It is assumed that the cells are dispersed in the gel at a density of $1.5x10^7$ cells/mL, which has been shown to be appropriate for nourishment of all βTC3 cells in hydrogel matrices of 1 mm thickness or less [138]. Thus, a volume of 0.2 mL accommodates the necessary number of cells; assuming the thickness of the gel to be 0.8 mm, the area of each flat surface is 2.5 cm^2.

---

[1] Computational calculations of the mathematical modeling were performed by Jeff Gross

The equations describing the function of the device are as follows.

$$\frac{d\left(VC_{G,in}\right)}{dt} = k_{eff}^{glu} A\left(C_{G,out} - C_{G,in}\right) - nVq_G \qquad (1)$$

$$\frac{d\left(VC_{I,in}\right)}{dt} = -k_{eff}^{ins} A\left(C_{I,in} - C_{I,out}\right) + nVq_I \qquad (2)$$

In the above equations, V is the volume inside the construct; $C_{G,in}$ ($C_{G,out}$) and $C_{I,in}$ ($C_{I,out}$) are the concentrations of glucose and insulin inside (outside) the construct, respectively; A is the surface area over which mass transport of glucose and insulin occurs; k_{eff}^{ins} (= D_{eff}^{ins}/L) and k_{eff}^{glu} (=D_{eff}^{glu}/L) are the effective permeabilities of insulin and glucose, respectively, via the glucose-responsive material; n is the cell density in the construct; and q_G and q_I are the specific rates of glucose consumption and insulin secretion, respectively.

In this study, q_G is assumed to follow Monod kinetics

$$q_G = \frac{v_{\max,G} C_G}{K_{m,G} + C_G} \qquad (3)$$

whereas q_I is constant for constitutively secreting cells. The values of all model parameters other than k_{eff} are given in Table 4.2.

Two parameters of major significance in determining the behavior of the system are (i) the insulin permeability through the material in the sol and gel states, and (ii) the kinetics of material change from gel to sol and vice versa upon stepping up and down the

glucose concentration. To generate the model simulations, an "idealized" material was considered, which differed from the current con A-glycogen material in the following two aspects:

1. The material was assumed to exhibit gel-sol transformations within the physiologic range of glucose concentrations. In contrast, the current con A-glycogen material needs to be exposed to higher than physiologic glucose concentrations to be converted from gel to sol. In particular, the material was assumed to have effective permeabilities to insulin k_{eff}^{ins} of 2.1 x 10^{-6} cm/s and 7.4 x 10^{-5} cm/s at 4 mM and 15 mM glucose, respectively. These are the values obtained with the existing con A-glycogen material sandwiched between the two polycarbonate membranes in experiments with cells (Fig. 7), except that the gel-sol changes occur at higher glucose concentrations.

2. The material was assumed to exhibit the same kinetics of transformation from sol to gel as from gel to sol. In contrast, the current con A-glycogen material is converted faster from gel to sol upon increasing the glucose concentration than it is from sol to gel upon decreasing the glucose concentration in the surrounding medium. The following equation was used to describe the kinetics of material transformation:

$$k_{new}(t) = k_{old}^{equ} + \frac{(k_{new}^{equ} - k_{old}^{equ})t}{K_{m,t} + t} \qquad (4)$$

where $k_{new}(t)$ is the dynamic response of the permeability in response to a glucose step change, k_{old}^{equ} and k_{new}^{equ} are the permeabilities at equilibrium before and after the glucose concentration change, respectively, and t is time. Assuming a value of 5 min for

parameter $K_{m,t}$ allows an approximate 90% phase change in 40 minutes, which has been observed experimentally for gel-to-sol tranformations. The dynamic response of the material permeability to insulin in response to a step up and a step down in glucose is shown in Figure 4.9. It should be pointed out that a material with such improved characteristics, although currently unavailable, is realistic, as will be discussed later in this article.

Model solutions were evaluated for a construct subjected to square waves of glucose concentration, from 4 mM for 4 hours to 15 mM for 2 hours back to 4 mM for 4 hours, and so on. Equations (1) and (2) were integrated from initial conditions

$C_{G,in}$ (t=0) = 0 and

$C_{I,in}$ (t=0) = 0

and with $C_{I,out}$ constant and equal to 0. Results for $C_{I,in}$ are shown in Figure 4.10A. As expected, there is initially net accumulation of insulin in the construct, but after transients have decayed $C_{I,in}$ oscillates between constant upper and lower limits (Figure 4.10B).

Table 4.2 Values of model parameters used in simulating the function of the device in Figure 4.8.

Parameter	Symbol	Value
Surface area of the membrane	A	2.5 cm^2
Cell density in construct	n	$1.5x10^7$ cells/ml
Maximum specific rate of glucose consumption (from [138])	$v_{max,G}$	$1.4\mu mol/(10^9 cells \cdot min)$
Saturation constant in Monod expression for glucose consumption rate	$K_{m,G}$	0.01 mM (from [138])
Specific rate of insulin secretion (constant)	q_I	$4x10^{-5}$ $\mu U/(10^5 cells \cdot min)$ (from [138])*
Volume of construct	V	0.2 mL

* The constitutively secreting cells considered in the mathematical simulations were assumed to secrete insulin at a rate similar to that of βTC3 cells exposed to high glucose.

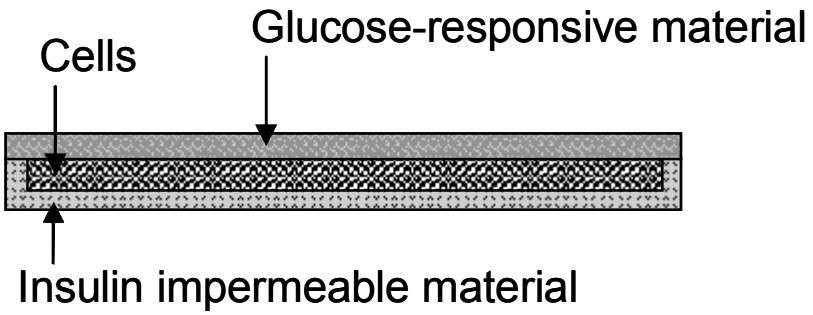


Figure 4.8 Cell-material hybrid construct configuration considered in the mathematical modeling simulations.

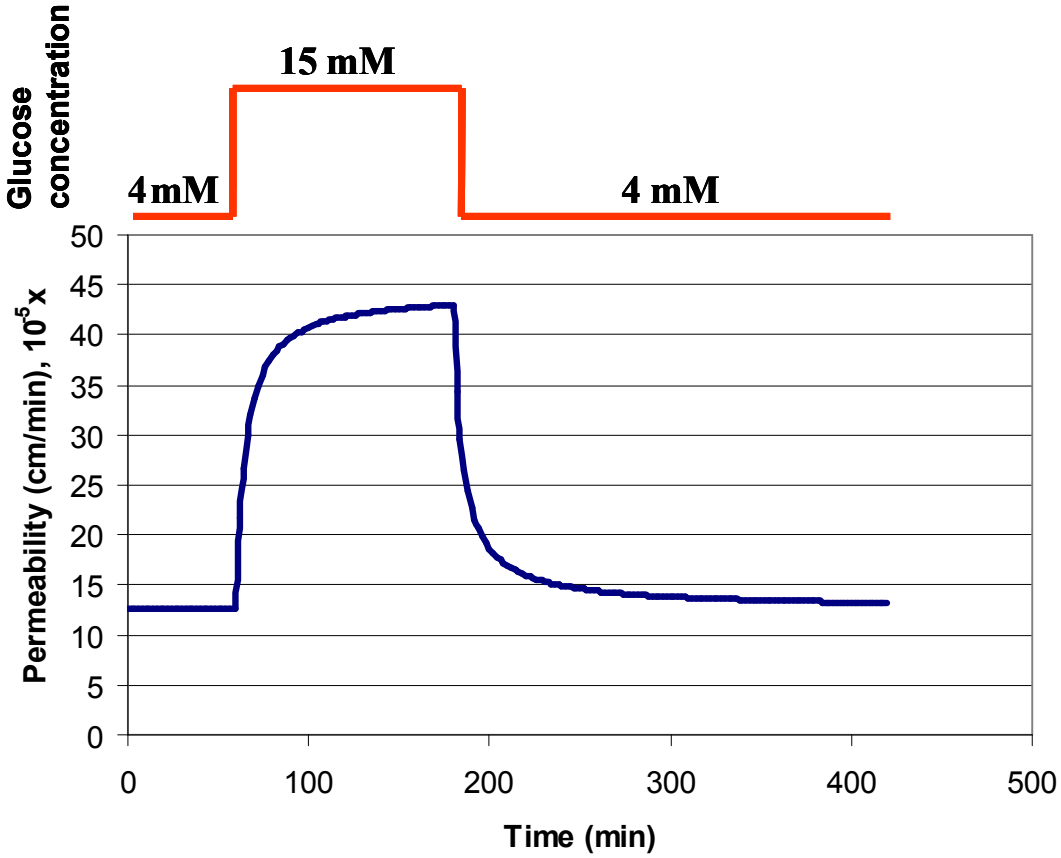
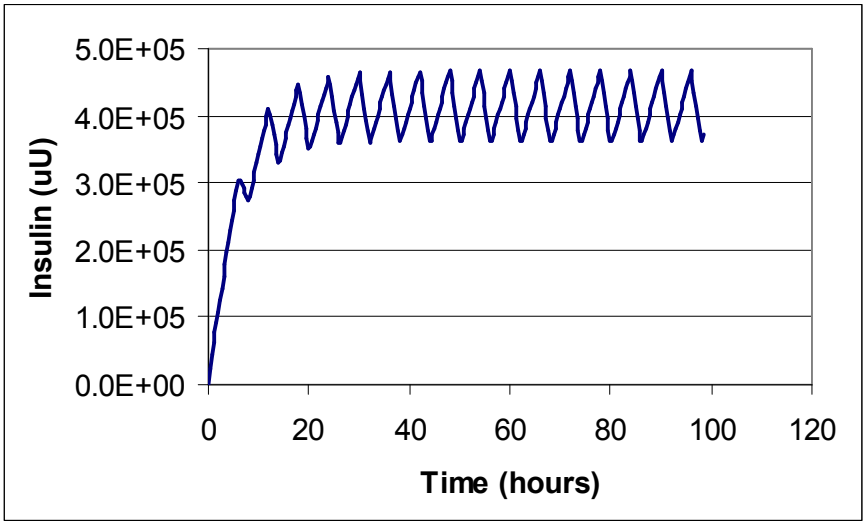


Figure 4.9 Kinetics of change of insulin permeability ($k_{eff}^{ins}=D_{eff}^{ins}/L$) through the material considered in the mathematical simulations when the glucose concentration is changed from 4 to 15 to 4 mM.

A



B

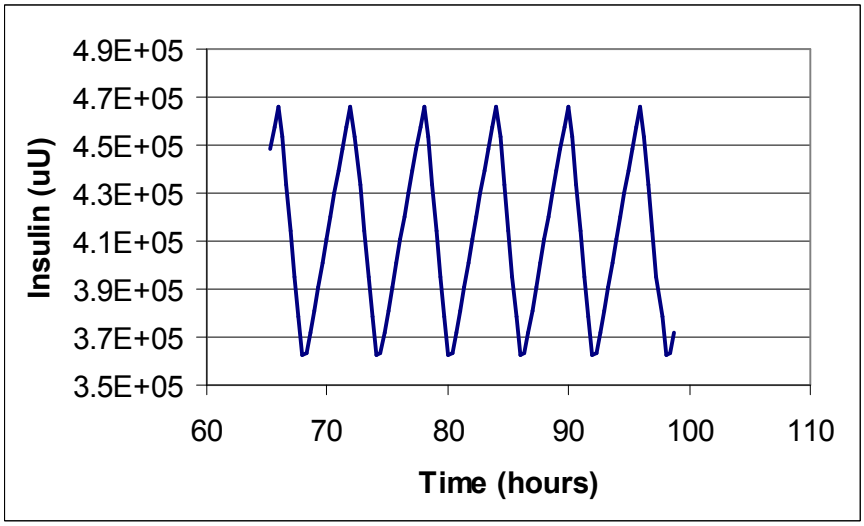


Figure 4.10 Function of the construct shown in Figure 4.8 subjected to square waves of 4 mM glucose for 4 hours and 15 mM glucose for 2 hours.
A. Accumulation of insulin in an initially insulin-free construct.
B. Oscillations of insulin concentration within construct after initial transients have decayed.

4.5 Discussion

A major challenge in developing a cell-based therapy for insulin-dependent diabetes consists of ensuring an appropriate cell source. Recent clinical studies treating diabetes in humans by transplantation of allogeneic islets have received significant attention [3, 139, 140]. However, the problems of limited tissue availability and of side effects of long-term immunosuppression remain as substantial obstacles in such a therapeutic approach. Autologous non-β cells, retrieved as a biopsy from the patient and genetically engineered for insulin secretion, constitute a promising alternative cell source. Engineering cells for insulin secretion is straightforward and such cells relax the problems of tissue availability and immune acceptance. However, introducing physiologic glucose responsiveness is problematic. Normal β cells exhibit a biphasic secretory response in response to a step up in glucose concentration. The first phase of secretion corresponds to the fast insulin release from granules and lasts 10 to 15 minutes. The second phase occurs over a period of hours and is related to elevated proinsulin biosynthesis. Insulin secretion stops quickly following a step-down in glucose level. Although it is unclear how closely a pancreatic substitute needs to mimic this type of secretion dynamics, genetically engineered non-β cells are generally far from this type of response, being either constitutive secretors of insulin or exhibiting glucose responsiveness but with sluggish dynamics [129-131, 133]. This paper presents a novel concept of a pancreatic substitute consisting of recombinant non-β cells and a material exhibiting glucose-responsive permeability to insulin which, as a whole, releases insulin with improved dynamics relative to the cells by themselves. Although the construct does not mimic the insulin secretion dynamics of normal β cells, it does represent an

improvement relative to insulin injections.

An ideal glucose-responsive material to be used in a hybrid device with cells should exhibit the following characteristics: It should be specific to glucose and not react with other molecules in bodily fluids, exhibit rapid kinetics in its transformation from one state to the other, and function under the physiologic environment, such as neutral pH. Additionally, it should be biocompatible or effectively enclosed in a semi-permeable barrier for biocompatibility, function with authentic insulin, i.e., it should not require insulin derivatization, and be stable in the long-term.

There exist three types of glucose responsive-materials reported in the literature [20, 141-143]. The first type, based on glucose oxidase, has received significant attention in drug delivery and the development of glucose sensors [20, 21, 144-146]. Glucose oxidase catalyzes the conversion of glucose to gluconic acid and hydrogen peroxide, thus lowering the pH when this reaction occurs. A hydrogel made from glucose oxidase and pH sensitive material may thus control the release of insulin by changing the structure of the pH-sensitive material when the reaction with glucose occurs. There exist, however, several drawbacks in using this type of material in a hybrid construct with cells. First, the solubility of natural insulin decreases as the pH is lowered [15]; second, this type of material exhibits long time lags, of the order of hours, in its response [21]; and third, the reaction causes depletion of oxygen and production of gluconic acid and hydrogen peroxide, which are known to inhibit the activity of glucose oxidase [147] and are possibly harmful to the cells as well.

A second type of material is based on phenylboronic acid, which can form a stable hydrogel with a polyol, such as poly(vinyl alcohol) [28, 29, 148, 149]. This hydrogel is

glucose-responsive, as it becomes dissociated in the presence of a competing polyol compound, such as glucose. Using insulin modified so as to contain polyol residues, a material that releases modified insulin in response to glucose is created [150]. Disadvantages of this approach include the need for derivatized insulin; and limited specificity towards glucose, as any polyol compound, such as plasma proteins and other saccharides, can react with phenylboronic acid.

The third type of glucose-responsive material is based on the glucose-binding protein con A, which is obtained from the jack bean plant. Con A generally binds to saccharides containing α-D-mannose or α-D-glucose residues and appears to recognize terminal as well as internal saccharide residues. Con A exists as tetramers under neutral pH, with the tetrameric complex having four binding sites towards glucose. When mixed with polysaccharides, such as glycogen and dextran, con A acts as a crosslinker forming a highly viscous gel. Free glucose in the surrounding solution can competitively displace the polysaccharide and dissociate the gel into a sol [35, 36, 134, 151]. In proof-of-concept studies on cell-material hybrid constructs, the con A-based material offers distinct advantages relative to the other glucose-responsive hydrogels for the following reasons: it is highly specific to glucose; it can function under physiologic environment; the kinetics of phase changes are relatively fast; the phase change is a reversible process; and it is not necessary to modify the insulin. Although con A is known to be immunogenic [32], the large molecular weight of the monomer (27,000) and especially of the tetramer that forms at neutral pH (108,000) enables the retention of the material in membranes, thus improving its biocompatibility.

The experiments of this study show that glucose can induce the phase change of

the con A-based material, and that the diffusivity of secreted insulin under high glucose concentration (sol state) is approximately 3.5 times higher than the diffusivity under low glucose concentration (gel state, Figure 4.7). The kinetics of gel to sol transformation are relatively fast, taking less less than 40 minutes in the apparatus of Fig. 1 to complete. On the other hand, the sol to gel phase change is slower. Plausible reasons for this include: (i) the possible leakage of some material, which may explain the higher diffusivity through the 2^{nd} sol state relative to the 1^{st} in the sol to gel to sol experiments (Figure 4.4); (ii) the intrinsically slower kinetics of formation of the gel network, relative to its breakdown, as the former involves the diffusion of glucose out of the material and the re-association of con A with the polysaccharide chains. Moreover, the possible entrapment of glucose in the material, following the change of the surrounding solutions, could delay the formation of the gel. It has been suggested that reaction by-products formed during material preparation, e.g. $B(OH)_3$, may become entrapped inside the hydrogel and affect the function of the material by slowing the kinetics of gel to sol transformation, however, such effects were not observed in this study.

The glucose concentration of 220 mM used to accomplish the gel to sol transformation is much higher than the upper limit of the physiologic glucose concentration of approximately 15 mM. The glucose concentration required for the phase change is expected to be different if con A is modified to alter its binding constant to glucose, or if another polysaccharide with different binding properties to con A is used [151, 152]. An example of the latter is offered by the work of Obaidat et al. [34], where with a synthetic polysaccharide the glucose concentration needed to achieve the phase change was 22 mM. Reducing the thickness of the material may improve the kinetics of

gel/sol transformations. Thus, by incorporating these modifications, a material could approximate the properties assumed in the modeling part of this work.

The membranes used to sandwich the material constitute another important aspect of the device design. The membranes should be biocompatible and have pores of a size that prevents leakage of glucose-responsive material components, while allowing transport of insulin without significant resistance. Polycarbonate membranes have been used in hemodialysis applications, and although such membranes may not be suitable for long-term implantation, they are adequate for proof-of-concept experiments, in which high insulin permeability with low con A leakage are needed. The 0.1 μm polycarbonate membranes did not completely prevent leakage of macromolecules, however, no toxic amounts of con A escaped over the course of experiments (Table 4.1). Such leakage may, however, result in significant immunogenicity *in vivo*. Modifications of the con A-base material, such crosslinking con A with polysaccharide or increasing molecular weight by peglayting con A, should increase retention of the material and improve the flexibility in selecting the membranes [38, 39].

In mathematical modeling simulations aimed at demonstrating that sequestration of constitutively secreting cells by a glucose-responsive material produces a construct with improved secretion dynamics, an idealized material was assumed. As previously discussed, it should be possible to improve both the glucose sensitivity and the transformation kinetics of the material by changing or derivatizing the macromolecules and by reducing the thickness of the sandwich configuration. According to the mathematical simulations, following the initial transients, the concentration of insulin inside the construct and the flux of insulin to the surroundings oscillated between

constant limits as the glucose concentration was cycled between 4 and 15 mM. If the cells in the construct secrete at a constant rate, as assumed in this model, then the overall rate of insulin release from the construct is appropriate for restoring normoglycemia in the mice, the animal for which the device was scaled. Incorporation of the material achieves a more physiologic regulation of insulin release, which occurs at a higher rate at high glucose than at low glucose concentration, as opposed to implanting the cells with an inert material, in which case insulin release would occur at a constant rate.

It should be noted that the model predicts insulin accumulation inside the device to concentrations of the order of 3.5×10^6 μU/ml, much higher than those in the surrounding environment. Experimentally, it has been found that insulin secretion from βTC3 cells is not feedback inhibited at insulin concentrations of 50,000 μU/mL (Tziampazis and Sambanis, unpublished results), and recombinant, constitutively secreting non-β cells are expected not to be feedback inhibited, either, at such insulin concentrations. However, it is unknown if the 70-fold higher insulin concentrations predicted to occur in the device have a negative effect on cells. Increasing the diffusivity through the glucose-responsive material and/or of the area through which insulin transport occurs decreases proportionately the insulin concentration in the construct.

Use of autologous cells offers significant flexibility in the type of material that can be used in an implantable device, as the cells do not need to be immunoprotected. The proof-of-concept studies presented in this paper indicate that it should indeed be feasible to associate constitutively secreting cells with a glucose-responsive material to generate a hybrid construct with insulin secretion dynamics closer to physiologic

compared to cells by themselves or encapsulated in an inert material, or compared to insulin injections. Clearly, improvements are needed on several aspects of the material function prior to *in vivo* experiments, including: (i) the material sensitivity should be improved towards the physiologic glucose range; (ii) the kinetics of gel-to-sol and, more so, of sol-to-gel transformations should be improved; and (iii) leakage of material components from the membrane sandwich should be minimized. The latter is expected to improve both the stability and biocompatibility of the preparation.

CHAPTER 5

TISSUE ENGINEERING A PANCREATIC SUBSTITUTE BASED ON GENETICALLY ENGINEERED CELLS AND GLUCOSE-RESPONSIVE MATERIAL

5.1 Abstract

Genetically engineered cells offer a solution to the cell availability problem in tissue engineering a pancreatic substitute for the treatment of insulin-dependent diabetes. These cells can be non-beta cells, such as hepatocytes or myoblasts, retrieved as a biopsy from the same patient and genetically engineered to secrete recombinant insulin constitutively or under transcriptional regulation. However, these cells exhibit sub-optimal insulin secretion dynamics and cannot, by themselves, restore normoglycemia in patients. Our objective consists of associating genetically engineered cells with a glucose-responsive material, where the latter acts as a control barrier for insulin release, thus realizing a cell-material hybrid with improved secretion characteristics. Proof-of-concept experiments were performed with insulinoma βTC3 cells, HepG2 hepatomas, and C2C12 myoblasts, the latter two genetically modified to constitutively secrete insulin. The control barrier consisted of concanavalin A (con A)-based glucose-responsive material. Results demonstrated that the device released insulin at a higher rate in response to glucose challenges; in contrast, a device containing an inert hydrogel instead of glucose-responsive material released insulin at an essentially constant rate, irrespective of the surrounding glucose concentration. The limitations of the proposed construct that need to be overcome prior to *in vivo* experiments are discussed.

5.2 Introduction

Insulin-dependent diabetes is a serious disease affecting more than 3 million people in the US. The current treatment commonly consists of frequent blood glucose monitoring and multiple daily insulin injections. However, this treatment does not provide the tight glycemic regulation afforded by a normally functioning pancreas, thus it cannot prevent long-term complications, which include cardiovascular disease, retinopathy and nephropathy [1, 9]. Closed-loop insulin delivery systems, consisting of a glucose sensor, a controlled pump, and an insulin reservoir, are promising at reducing the occurrences of hypo- and hyperglycemia. However, the clinical application of such systems is still held back by technical difficulties, primarily the *in vivo* longevity and stability of glucose sensors [14].

Cell-based therapies have significant potential in correcting the drawbacks of the insulin delivery systems mentioned above. Transplantation of human islets from cadaveric donors following the Edmonton protocol, which uses steroid-free immunosuppressive drugs, results in insulin independence for 80% of the treated patients over a period of 12 months [55]. However, as the number of available donors will never meet the large number of patients, and the risk of life-long immunosuppression is of concern [153], immunologically acceptable insulin-producing cells that exhibit proper secretion dynamics need to be sought. Non-β cells, such as fibroblasts, myoblasts, and hepatocytes, can be retrieved as a biopsy from the same patient and engineered to express recombinant insulin [7, 83, 84, 154]. Due to their autologous nature, such cells relax the immune acceptance challenge, however, their insulin secretion is either constitutive or regulated at the transcriptional level, thus constant or sluggish in response to physiologic

stimuli and inadequate for glycemic regulation in higher animals and humans.

Instead of relying on cellular and molecular tools, we earlier proposed the concept of improving the secretion profile of such β cell surrogates by sequestering them in a glucose-responsive material [155]. At low glucose concentrations, the material is in a gel state that exhibits low insulin permeability, whereas at high glucose levels the material becomes a sol that exhibits a higher permeability to insulin [134, 155]. Thus, at low glucose concentrations, a higher fraction of the insulin secreted by cells accumulates within the construct, and the insulin release rate is relatively low. When the construct is exposed to high glucose, the material becomes a more permeable sol, and the insulin release rate from the construct is higher. As the time constant of the gel to sol and of sol to gel transformations is shorter than the (infinite) time constant of constitutively secreting cells or the (long) time constant of transcriptionally regulated cells, the kinetics of material transformation determine the kinetics of insulin release from the entire device.

In this article, we report the development of a disk-shaped prototype incorporating as part of its boundary a concanavalin A (con A)-based glucose-responsive material and containing one of three different cell types: mouse insulinoma βTC3 cells, human HepG2 hepatomas, and murine C2C12 myoblasts, the latter two genetically engineered for constitutive insulin secretion. Control constructs were fabricated in the same fashion, except that an inert material was used instead of the conA-based barrier. Insulin release from the devices was characterized by subjecting them to two square waves of glucose, each lasting two hours. The potential of such cell-material hybrid constructs in treating diabetes and the limitations that need to be overcome prior to *in vivo* applications are discussed.

5.3 Materials and Methods

5.3.1 Glucose-Responsive Material

The preparation of con A-glycogen material was based on the published protocol by Taylor et al [134]. Briefly, glycogen (Sigma Chemical Co., St. Louis, MO) was dissolved in PBS at 60% (w/v) and mixed with a 0.2M $NaIO_4$ solution at 1:1 volume ratio for 24 hours in the dark. The hydrogel was formed by mixing the resultant glycogen solution with a 15% w/v con A (Amersham Biosciences, Piscataway, NJ) solution in PBS at a 1:1 volume ratio. The gel was left at room temperature 24 hours followed by 1 hour immersion in 1 mg/ml $NaBH_4$ at 4°C. Finally, the gel was washed at least three times with PBS buffer and stored in PBS at 4^oC for later use.

5.3.2 Cell Culture and Transfection

βTC3 cells were obtained from the laboratory of Shimon Efrat, Albert Einstein College of Medicine, Bronx, NY. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 15% horse serum (Sigma), 2.5% fetal bovine serum (Hyclone, Logan, UT), 1% penicillin/streptomycin (Mediatech, Herndon, VA), and L-glutamine (Gibco, Grand Island, NY) to a final concentration of 6 mM. HepG2 human hepatoma cells (ATCC, Manassas, VA, USA) and C2C12 mouse skeletal myoblasts (ATCC) were cultured in DMEM (Mediatech) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (Mediatech). C2C12 myoblasts were induced to differentiate into myotubes by culturing in low serum medium consisting of DMEM (Mediatech) supplemented with 2.5% hourse serum (Hyclone) and 1% penicillin/streptomycin (Mediatech) [7]. Myotube formation was observed under the

microscope 3 days after the cells were switched to the low serum medium. In this study, C2C12 cells were transfected as myoblasts.

The plasmid, CMV/ Furin-B10 PPI cDNA/ Puro^R, used to engineer HepG2 and C2C12 cells into stable insulin-secreting cells, was constructed as previously described by Tang and Sambanis [86] and is shown in Figure 5.1A. Briefly, a DNA fragment containing IRES and puromycin resistance genes from plasmid pIRESpuro (Clontech, Palo Alto, CA) was connected to the 3'-end of the Human preproinsulin (PPI) cDNA in the vector, CMV/ Furin-B10 PPI cDNA (Figure 5.1B). The PPI cDNA was furin-compatible with a His B10-to Asp mutation (B10 mutation), and was a kind gift of Genetech, San Francisco, CA [72]. The expression cassette of plasmid CMV/ Furin-B10 PPI cDNA/ Puro^R generated a bicistronic mRNA and directed the synthesis of both the insulin and puromycin resistance protein, thus facilitating the selection process of stable colonies.

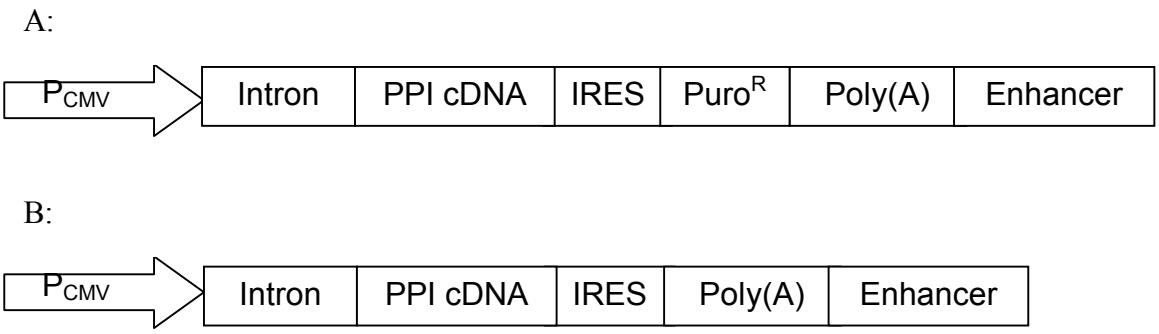


Figure 5.1 Plasmid structures
A: CMV/ Furin-B10 PPI cDNA/ Puro^R.
B: CMV/Furin-B10 PPI cDNA from [86].

When HepG2 and C2C12 cells reached about 60% confluency, they were treansfected using the FUGENE 6 reagent (Roche, Indianapolis, IN) according to the manufacturer's protocol. In a T-75 flask, cells were incubated 24 hours with the culture medium (without antibiotics) containing the plasmid (15 μg) and the FUGENE 6 reagent (45 μl). After transfection, cells continued to be cultured for recovery until they were ready to be split. When the transfected cells reached about 90% confluency, cells were trypsinized and split in 1:100 ratios into 100 mm culture dishes. During the selection process, which lasted over one month, cells were cultured in the presence of 2 μg/ml puromycin. Only cells that expressed the puromycin resistance gene survived under the selection pressure. Surviving cells formed colonies, which could be clearly seen under a microscope, then cells were retrieved by cloning disks and plated into the wells of a 24-well plate. In all subsequent experiments, engineered cell lines were cultured in media supplemented with 1 μg/ml puromycin.

5.3.3 Cell Encapsulation

To facilitate cell loading into hybrid constructs, βTC3 cells and recombinant HepG2 and C2C12 cells were encapsulated in 2% alginate (ISP Inc., San Diego, CA) at a density of $7x10^7$ cells/ml of alginate following the general protocol published by Stabler et al [71], except that a poly-L-lysine membrane and a final alginate coating were not applied. Briefly, cells from confluent monolayer cultures were detached by EDTA-trypsin (Sigma). A sample of the cell suspension was used for cell counting with trypan blue (Sigma); the rest of the suspension was centrifuged for 4 minutes at 110 g, and pellets were mixed with sodium alginate sterilized by filtration through a 0.2-μm

syringe filter (Pall Life Sciences, Ann Arbor, MI). Alginate beads of approximately 700 μm diameter were formed by passing the cell-alginate suspension through an electrostatic bead generator (Nisco Engineering Inc., Zurich, Switzerland) into a 1.1% $CaCl_2$ solution. Beads were washed twice with culture medium, incubated overnight on an orbital shaker, and loaded in constructs for secretion experiments the following day. To ensure that recombinant HepG2 and C2C12 cells were functional once encapsulated, beads were also cultured in T-flasks on a rocking platform for up to 10 days with medium changes every one to three days, depending on the cell growth rate. At different times, bead samples were withdrawn for histology and viability assessment by trypan blue staining as described in the analytical techniques. As βTC3 cells have been shown to retain their function after alginate encapsulation [71], T-flask cultures of alginate beads with these cells were not performed.

5.3.4 Construct Design and Fabrication

The geometry of the cell-material hybrid construct is shown in Figure 5.2. Two polycarbonate plates (3 mm in thickness, 6 cm in diameter), each with a 3.7 cm diameter hole in the center (see the top view of the construct in Figure 5.2), constituted the main structural components of the construct. Two silicon sheets (2 mm in thickness, 4.7 cm in diameter), also each with a 3.7 cm diameter hole in the center, were used to separate the membranes that enclosed the glucose-responsive material and the cells. The material was sandwiched between one 0.02 μm pore size Anodisc membrane (Whatman Inc., Clifton, NJ), positioned between the material and the cells, and one 0.1 μm pore size polycarbonate membrane (Whatman Inc.), positioned between the material and the

surrounding medium. Both of these membranes are permeable to insulin. To minimize leakage of con A towards the cell compartment, while not excessively restricting diffusion of insulin through the membranes themselves, the smaller pore size membrane was used to separate the cells and the material and the larger pore size membrane was placed between the material and the surrounding medium. The cell compartment was sequestered on the other side by a polyethersulfone membrane of 3kDa molecular weight cutoff (Pall Life Sciences), which allowed passage of small molecules, such as glucose and oxygen, for cellular nourishment, but effectively excluded insulin.

In experimental constructs with glucose-responsive material, pieces of con A-glycogen gel of approximately 2 ml total volume were transferred by a spatula and used to fill the hole of the silicon sheet on top of the Anodisc membrane. Then, the polycarbonate membrane was positioned on top of the material to complete the material barrier. Two types of control constructs were fabricated, one with calcium alginate and the other with culture medium in place of con A-glycogen. In the alginate control, the material compartment was first assembled with the Anodisc and polycarbonate membranes and the polycarbonate plates sandwiching the silicon sheet tightly. Sodium alginate (ISP Inc.), filter-sterilized through 0.2 μm syringe filter (Pall Life Sciences), was then injected, filling the space in between the membranes. The whole construct was immersed in a 1.1% $CaCl_2$ solution for 30 minutes to ensure gelation of the alginate inside the barrier. For the control construct with culture medium, the medium was injected into the material compartment after the entire construct was assembled.

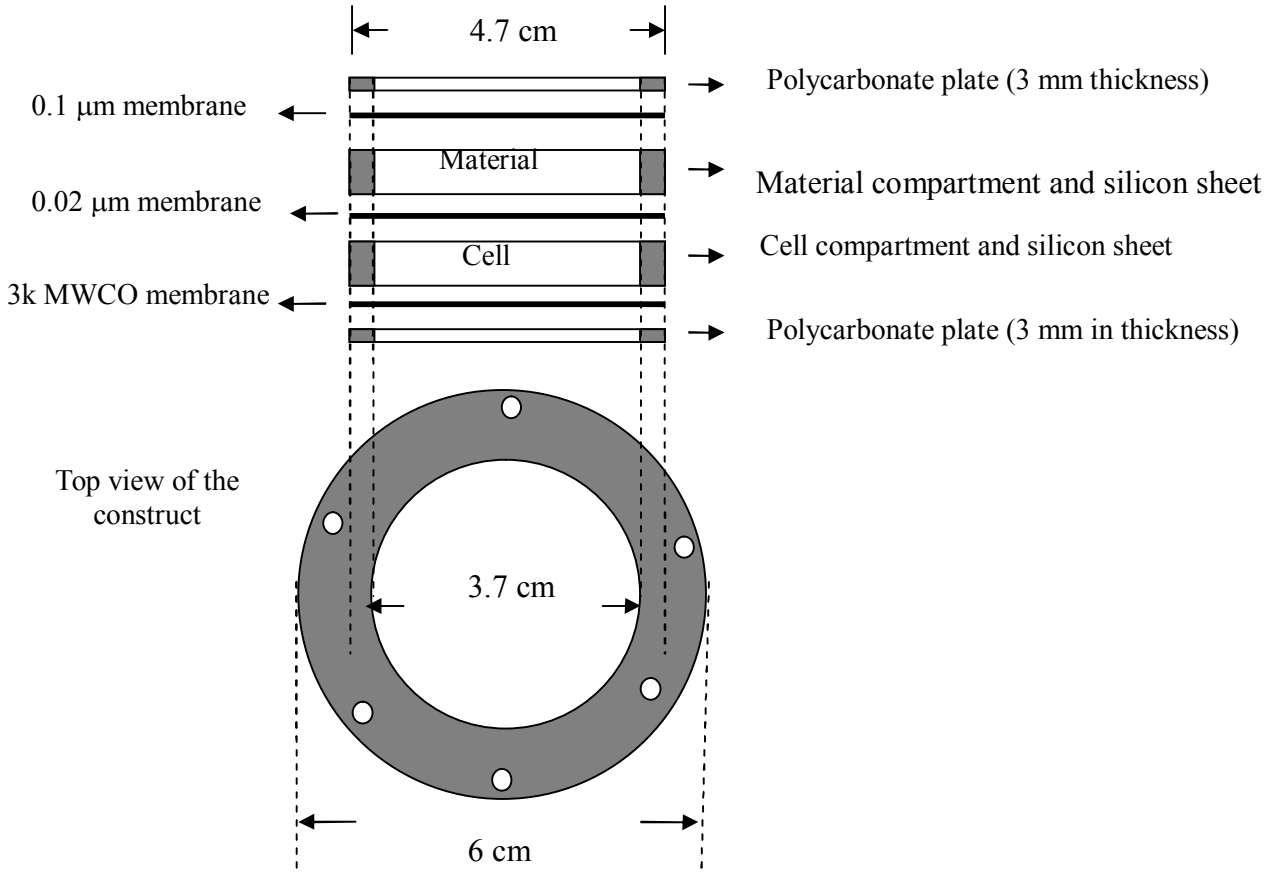


Figure 5.2 Cell-material hybrid construct configuration. The con A-glycogen material was placed in the 3.7 cm diameter hole of a 2 mm-thick silicon sheet and was sandwiched between a 0.1 μm pore-size polycarbonate and a 0.02 μm pore-size Anodisc membrane. The cell compartment consisted of alginate-encapsulated insulin-secreting cells positioned in the 3.7 cm diameter hole of a 2 mm-thick silicon sheet and bounded on one side by the material compartment and on the other side by a 3k Dalton MWCO polyethersulfone membrane. Polycarbonate plates with 6 screws were used to fix everything in place. Control constructs were fabricated in the same way, except that calcium alginate or plain culture medium was used instead of con A-glycogen. The figure is not drawn to scale.

To assemble the cell compartment, 1 ml of alginate-encapsulated insulin-secreting cells were first placed on the top of the 3 kDa MWCO cellulose ester membrane on the lower polycarbonate plate. The material sandwich assembled as described previously was then laid on top of the cell compartment, followed by the material-side polycarbonate plate, and the whole construct was tightly assembled by screws. Approximately 1 ml of the culture medium was then injected into the cell compartment. The assembled construct was immediately immersed in a beaker containing 50 ml of culture medium with 25 mM glucose and supplemented with serum, as described in the cell culture section for the different cell lines. The beaker and the construct were then placed inside a tissue culture incubator on top of an orbital shaker that provided adequate mixing.

5.3.5 Characterization of Insulin Release from Constructs

Each construct was cultured for 20 hours prior to glucose concentration changes to allow insulin to accumulate inside the cell compartment. Two square-waves of glucose were implemented to test the glucose responsiveness of the hybrid construct. Immediately prior to the glucose changes, the culture medium was replaced with fresh, so when an experiment started at t=0, the surrounding medium was insulin-free and contained 25 mM of glucose. Following incubation for 2 hours in this medium, 2.0 g of glucose was directly added to the beaker to increase the glucose concentration to 220 mM. For the glucose step down 2 hours later, the medium was completely replaced with fresh culture medium containing 25 mM glucose, and the cycle was repeated once more. Samples were collected every 30 minutes during experiments, and the same amount of culture medium was added back to maintain constant volume inside the beaker. All

samples were stored at -20°C for later insulin assay. At the end of the experiment, the constructs were disassembled, and the alginate-encapsulated cells were collected to assess cell viability.

5.3.6 Analytical Techniques

5.3.6.1 Cell Viability

To measure the viability of alginate-encapsulated cells, beads were dissolved in a 2.2% sodium citrate solution, and the resulting cell suspension was centrifuged for 3 minutes at 110 g. Pelleted cells were re-suspended in culture medium and mixed with trypan blue (Sigma) at 1:1 volume ratio. Cell viability in the final suspension was determined on a hemocytometer under a microscope by trypan blue exclusion.

5.3.6.2 Insulin Assay

Insulin secreted by βTC3 and HepG2 cells was assayed by rat insulin radioimmunoassay (Linco Research, St. Charles, MI, USA). The antibody used in the assay had a 100% reactivity against mouse and human insulin and a 69% cross-reactivity against human proinsulin. Insulin secreted by C2C12 cells was assayed by a human-specific insulin radioimmunoassay, which had 0.02% cross-reactivity to human proinsulin. This ensured that the immunoreactive insulin secreted by C2C12 cells was mature insulin.

5.3.6.3 Histology

For histology, alginate beads were fixed with a 3% glutaraldehyde solution, then

embedded in paraffin blocks. Blocks were later sectioned by a microtome, and the obtained slides were stained with hematoxylin/eosin (H/E).

5.4 Results

5.4.1 Construct with βTC3 cells

Results with constructs consisting of βTC3 cells and glucose-responsive material (experiment) or culture medium (control) are shown in Figure 5.3. βTC3 cells are glucose-responsive but hypersensitive, as they express the GLUT-1 instead of the GLUT-2 glucose transporter and hexokinase instead of glucokinase [5, 68]. Thus, the cells secreted insulin constitutively under the 25-220 mM glucose used in this experiment. The average insulin release rate over each indicated time period was calculated from the slope of the least squares fit of the released insulin concentration vs. time data; each rate was normalized to the corresponding low glucose release rate during the 0-2 hour period, which was set equal to 1. For the experimental construct, the insulin release rates changed with the step changes of glucose: the rates during high glucose were, on average, 2- to 3- fold higher than those during the low glucose periods. On the other hand, insulin release rates from the control construct did not respond to changes in the surrounding glucose level.

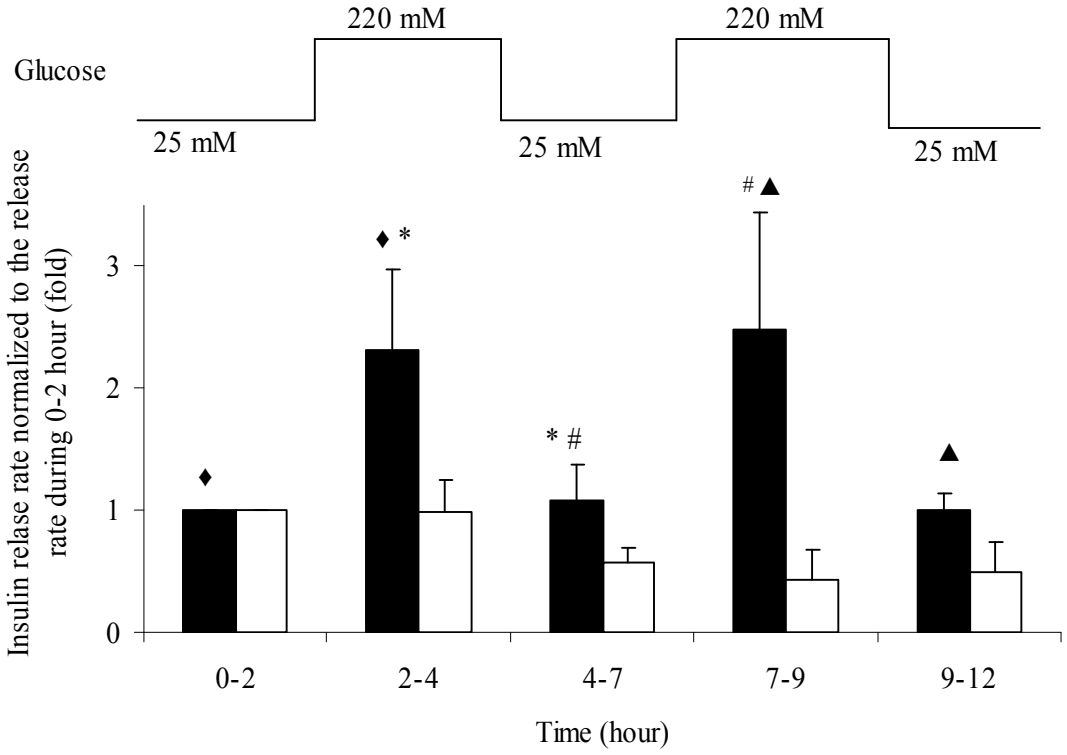


Figure 5.3 Insulin release profiles from hybrid construct of alginate-encapsulated insulin-secreting βTC3 cells with con A-based material (experiment, ■) and with culture medium (control, □). In each independent experiment, insulin release rates were normalized to the rate during 0-2 hour, which was set equal to 1. ♦, *, #, and ▲ indicate pair-wise statistical comparisons using a one-tailed t-test, $p<0.05$. Error bars indicate standard deviation. N=3 for all.

5.4.2 Construct with genetically engineered non-β cells

An autologous cell therapy approach for insulin-dependent diabetes should be based on non-β cells that can be retrieved as a biopsy and genetically engineered to secrete insulin. Such cells include hepatocytes, retrieved as a biopsy from the liver, and myoblasts, retrieved as a biopsy from muscle tissue. In proof-of-concept experiments, human HepG2 hepatomas and mouse C2C12 myoblasts were genetically engineered for constitutive expression of insulin, encapsulated in calcium alginate beads and associated with glucose-responsive material (experiment) or with calcium alginate (control) in the disk-shaped device shown in Figure 5.2. The construct responses to step changes in glucose concentration were then evaluated.

5.4.2.1 Construct with HepG2 cells

HepG2 cells were genetically engineered by chemical transfection with the CMV/ Furin-B10 PPI cDNA/ $Puro^R$ plasmid shown in Figure 5.1A. Clones that survived under the selection pressure secreted insulin constitutively at a rate of 9.5±2.3 μU/(hour•10^5 cells). Cells were encapsulated at a density of $7x10^7$ cells/ml of alginate prior to incorporation in the hybrid construct. Histology sections of alginate encapsulated cells propagated in T-flasks on an orbital shaker are shown for different time points in Figure 5.4. Cells proliferated in the alginate beads, and since no poly-L-lysine coating was applied, beads became unstable and cells started protruding out of them by day 9. This phenomenon was not observed with beads incorporated in constructs, as all construct experiments were terminated by day 3 after bead preparation. Additionally, trypsinization and encapsulation had no short-term effect on insulin secretion from

HepG2 cells, as the per-cell secretion rate from calcium alginate-entrapped cells two days post-encapsulation was the same as the secretion rate from monolayer cultures.

Results from experimental and control constructs with HepG2 cells are shown in Figure 5.5. In the control construct, insulin-release rates stayed at the same level through the two glucose square-waves in the surrounding environment ($P>0.1$ between high and low glucose periods). On the other hand, in the experimental construct, insulin-release rates responded to changes in the surrounding glucose concentration. In fact, the average rates showed an 8-fold difference between high and low glucose. Contrary to the experiment with βTC3 cells, in the experiment with recombinant HepG2 cells and the glucose-responsive material the insulin-release rate during a 25 mM glucose period was higher than the rate during the prior 25 mM glucose period. For example, the rate during the 4-7 hour period was higher than that during the 0-2 hour period ($p<0.001$). This appeared to be due to degradation and loss of con A-glycogen material exaggerated by the presence of the high FBS concentration in the HepG2 culture medium. This possibility was further investigated in experiments with C2C12 cells (see below). Even though the material was not able to completely revert to the original gel state after lowering the glucose level, the insulin release rate during a 25 mM glucose period was always statistically lower than that during the previous 220 mM glucose period (e.g. $p<0.05$ between the rates for hours 2-4 and 4-7).

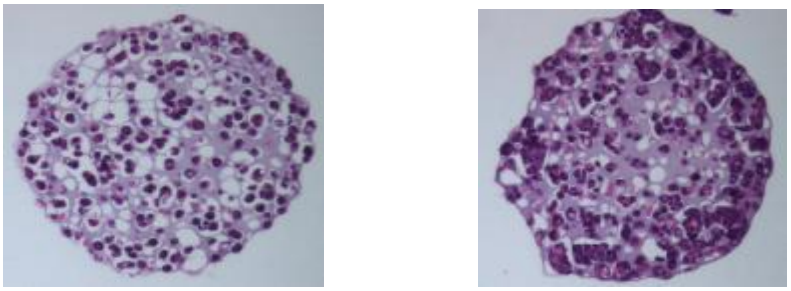


Figure 5.4 Representative histology cross-sections of alginate-encapsulated HepG2 cells on days 3 and 6 post-encapsulation. Cells were encapsulated at an initial density of $7x10^7$ cells/ml of alginate in 700 μm diameter beads.

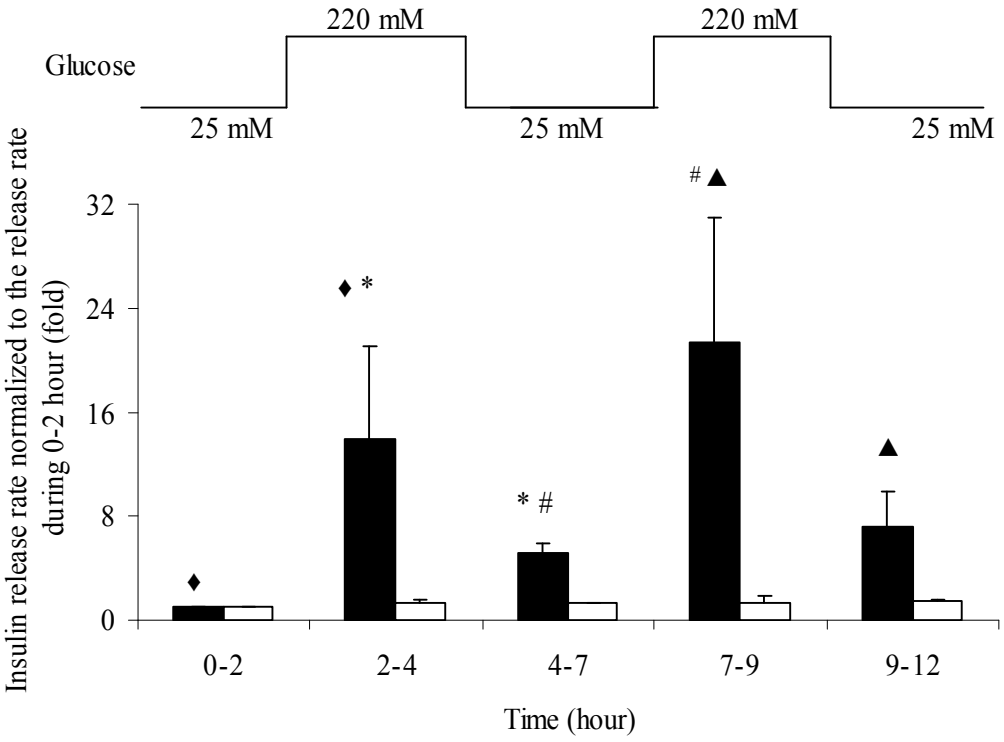


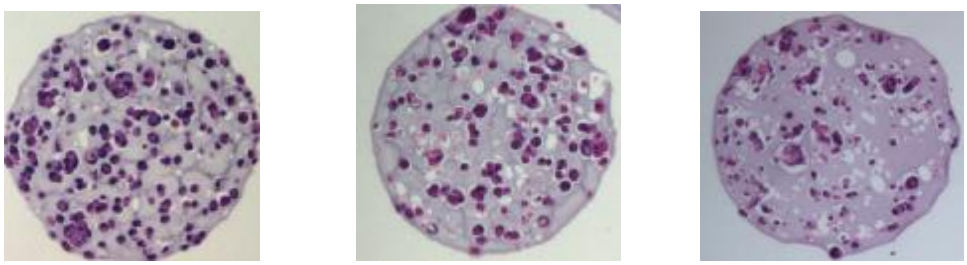
Figure 5.5 Insulin release profiles from hybrid construct of alginate-encapsulated insulin-secreting HepG2 cells with con A-based material (experiment, ■) and with alginate (control, □). In each independent experiment, insulin release rates were normalized to the rate during 0-2 hour, which was set equal to 1. ♦, *, #, and ▲ indicate pair-wise statistical comparisons using a one-tailed t-test, $p<0.05$. Error bars indicate standard deviation. N=4 for all.

5.4.2.2 Construct with C2C12 cells

Stable C2C12 clones isolated under antibiotic selection secreted recombinant insulin constitutively when cultured as undifferentiated myoblasts or as differentiated myotubes in monolayer cultures. The insulin-secretion rate from undifferentiated C2C12 myoblasts was 17.5±3.4 μU/hr-10^5 cells, whereas C2C12 myotubes secreted insulin at a rate of 43.4±9.5 μU/hr-10^5 cells. The higher insulin-secretion rate from myotubes relative to undifferentiated C2C12 cells is consistent with literature reports [96]. C2C12 were encapsulated in alginate at a density of $7x10^7$ cells/ml of alginate and were propagated in either FBS or HS-supplemented culture media for 10 days. Histology sections of beads at different time points are shown in Figure 5.6. Unlike HepG2 cells, C2C12 cells proliferated slowly after encapsulation. C2C12 cells are reportedly unable to differentiate when plated on alginate sheets [72] and remained undifferentiated in alginate beads in this study, too. Consequently, their growth patterns were similar in the FBS and HS-supplemented media, as indicated by histology. Alginate entrapped C2C12 cells secreted insulin post-encapsulation in both types of culture medium used, and the per-cell secretion rate was 9.1±1.7 μU/hr-10^5 cells, lower than rates measured in monolayer cultures.

Although C2C12 cells were not able to differentiate in alginate via exposure to low-serum medium, construct experiments were still performed with both 10% FBS and 2.5% HS-supplemented media to further investigate whether a high FBS concentration caused the con A-glycogen degradation observed with HepG2-containing constructs. Insulin-release profiles from the C2C12-based constructs are shown in Figures 5.7 and 5.8. Insulin release rates from control constructs were at the same level in high and low

glucose with both types of serum. On the other hand, the insulin release rates from experimental constructs responded to changes in glucose concentration, but the degree of induction differed with type of culture medium. With 2.5% HS-supplemented medium, the insulin release rates under low glucose remained constant ($P>0.3$ between values at 4-7 hours and 9-12 hours), and the insulin release rate under high glucose was, on average, 5-fold higher than the corresponding low glucose rate (Figure 5.7). This result correlated well with the experiments with βTC 3 cells (Figure 5.3), in which the culture medium contained 2.5% FBS and 15% HS. With the 10% FBS-supplemented medium, results (Figure 5.8) were similar to those obtained with the HepG2-containing constructs, in which the same type of medium was used (Figure 5.5). Insulin release rates during the high glucose periods were all higher than that the rates during the low glucose periods, which demonstrated that the release of insulin from the experimental construct was glucose-dependent. However, the insulin release rates during the 25 mM glucose periods increased with time in the experiment: the rate during the 4-7 hour period was 3.3-fold higher than the rate during the 0-2 hour period; and the rate during the 9-12 hour period was 5.5-fold higher than that during the first low glucose period. Furthermore, insoluble particulate matter was observed in the containers at the end of the 20 hour incubation period prior to the glucose concentration step changes only when the high FBS medium was used with both the HepG2 and C2C12 cells as insulin source. Thus, it appears that the high concentration of FBS exaggerated the degradation and loss of con A-based material from the membrane sandwich.



Day 2 in 10%FBS Day 7 in 10% FBS Day 7 in 2.5% HS

Figure 5.6 Representative histology cross-sections of alginate encapsulated insulin-secreting C2C12 cells on days 2 and 7 for cultures in medium with 10% FBS and on day 7 for cultures in medium with 2.5% HS. Cells were encapsulated at an initial density of $7x10^7$ cells/ml of alginate in 700 μm diameter beads.

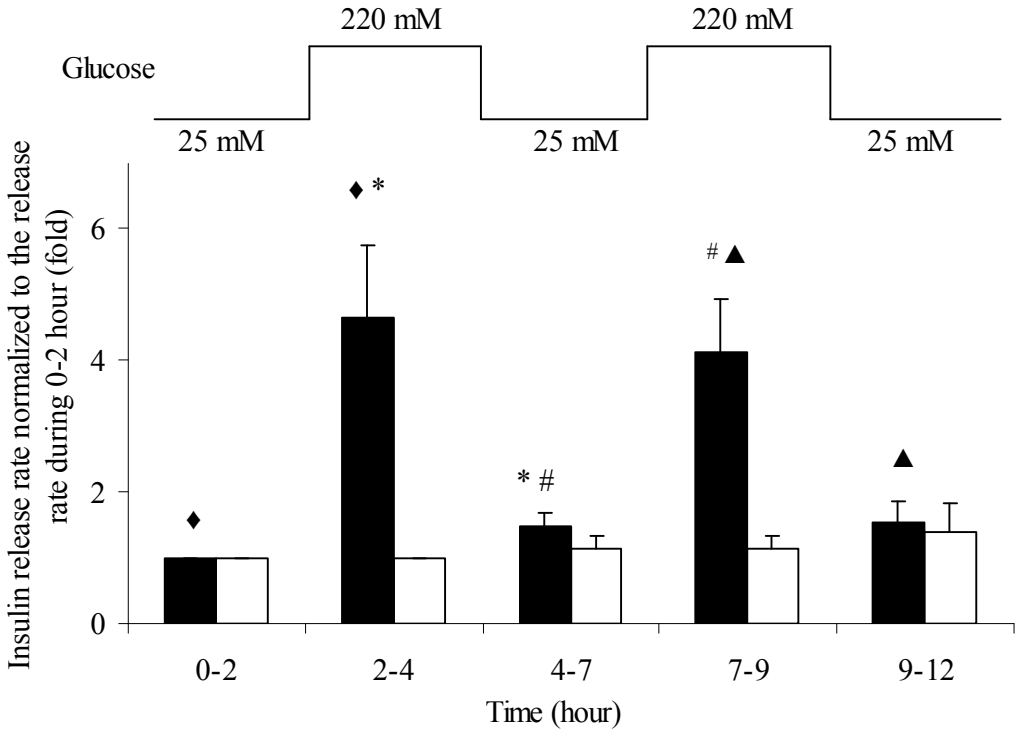


Figure 5.7 Insulin release profiles from hybrid construct of alginate-encapsulated insulin-secreting C2C12 cells with con A-based material (experiment, ■) and with alginate (control, □). Media used were supplemented with 2.5% HS. In each independent experiment, insulin release rates were normalized to the rate during 0-2 hour, which was set equal to 1. ♦, *, #, and ▲ indicate pair-wise statistical comparisons using a one-tailed t-test, $p<0.05$. Error bars indicate standard deviation. N=3 for all.

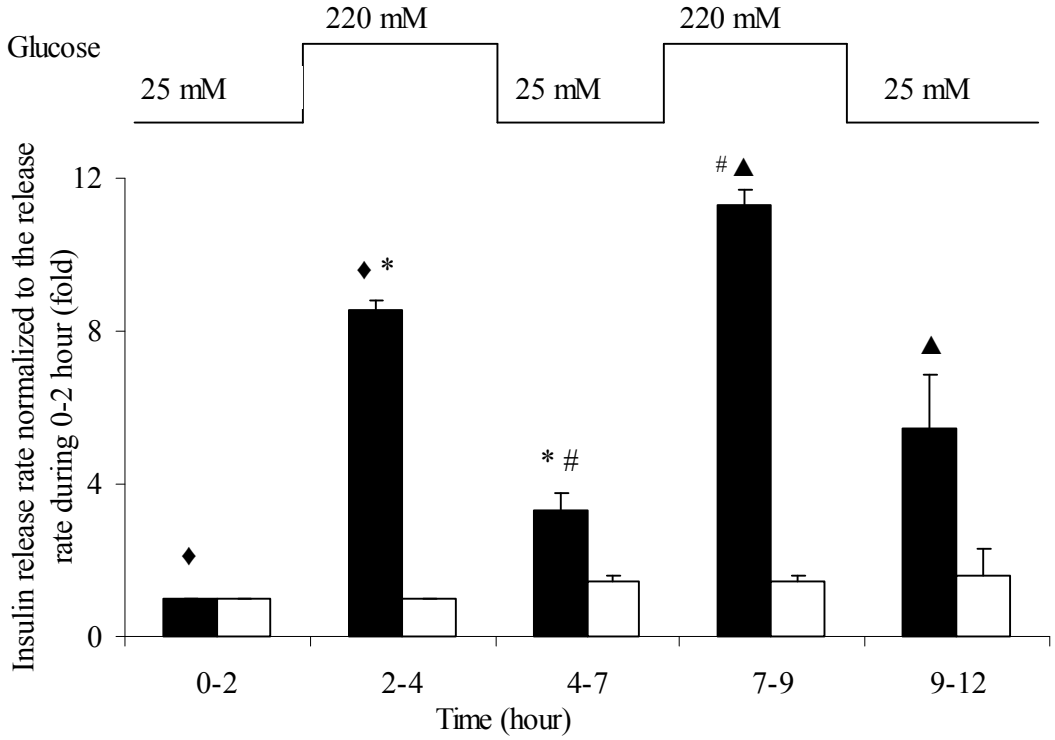


Figure 5.8 Insulin release profiles from hybrid construct of alginate-encapsulated insulin-secreting βTC 3 cells with con A-based material (experiment, ■) and with alginate (control, □). Media used were supplemented with 10% FBS. In each independent experiment, insulin release rates were normalized to the rate during 0-2 hour, which was set equal to 1. ♦, *, #, and ▲ indicate pair-wise statistical comparisons using a one-tailed t-test, $p<0.05$. Error bars indicate standard deviation. N=3 for all.

5.4.3 Cell Viability

The viabilities of different cell lines in experimental and control constructs are shown in Figure 5.9. In control constructs, βTC3 and C2C12 cells maintained viabilities above 60% at the end of 32 hour-long tests. However, the viability of HepG2 cells in control constructs dropped to below 50% by the end of tests, which suggested that HepG2 cells were more sensitive to the sub-optimal environment inside constructs. Cell viabilities in experimental constructs were statistically lower than those in the corresponding control constructs.

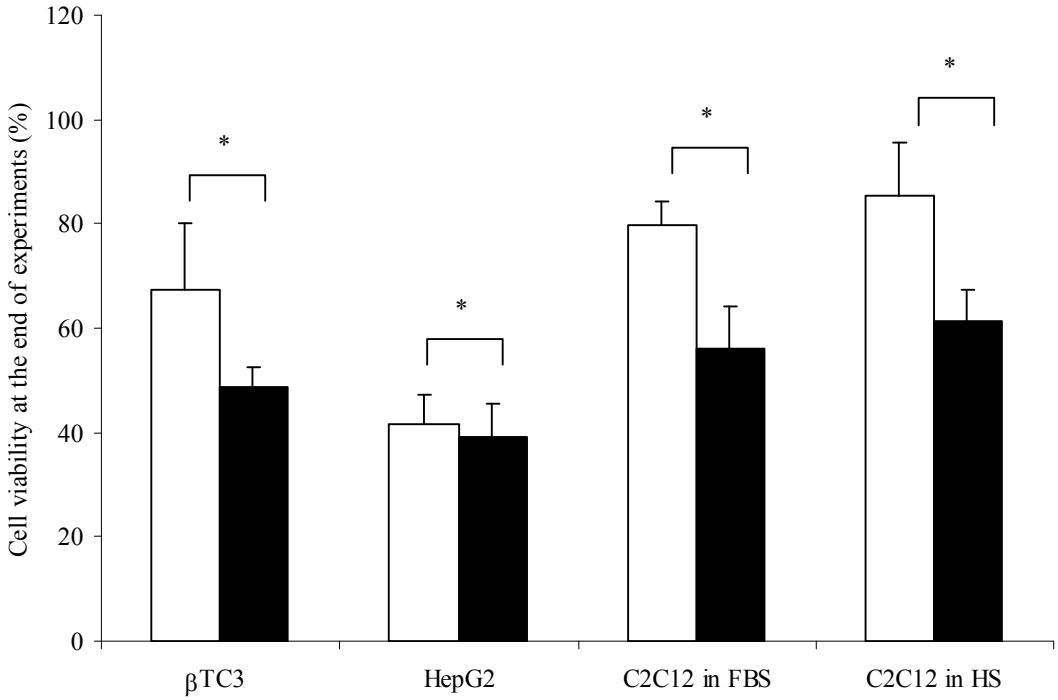


Figure 5.9 Viabilities of alginate-encapsulated cells at the end of construct experiments with con A-based material (experiment, ■) and with culture medium (βTC 3 cells (n=3)) or alginate (HepG2 (n=4) and C2C12 (n=3) cells) (control, □). * indicate pair-wise statistical comparisons using a one-tailed t-test, $p<0.03$. Error bars indicate standard deviation.

5.5 Discussion

Glycemic regulation in higher animals and, eventually, humans requires delivery of insulin in close response to physiologic needs. Normal islets accomplish this regulation through control of both insulin biosynthesis and secretion by glucose and other metabolic signals. In response to a step up in glucose concentration, islets exhibit a biphasic secretory response. The rapid first phase of insulin secretion results from the release of pre-synthesized insulin already stored in secretory granules; the second, more prolonged phase of secretion is due to elevated insulin biosynthesis and/or the preparation of another group of granules for release [52, 53].

Non-β cells that can be retrieved as a biopsy from a patient can be engineered to express recombinant insulin but generally lack a system of secretory granules that is responsive to glucose. Introducing regulation at the transcriptional level via the use of glucose-responsive promoters is helpful, but the resulting cells still cannot release insulin acutely in response to a glucose challenge [83, 84, 132, 156]. In this work, we developed and characterized a prototype consisting of a constitutively secreting β cell line or recombinant non-β cells sequestered in a glucose-responsive material. We demonstrated that the insulin released from the prototype was responsive to glucose challenges, whereas if an inert material was used, the insulin release rate did not respond to the same glucose concentration changes.

Among non-β cells, hepatocytes and myoblasts are particularly promising for the development of β-cell surrogates. Hepatocytes can be obtained as a biopsy, they express GLUT2 and glucokinase, two major glucose-sensing components in β cells that are involved in regulating insulin secretion, and hepatic expression of insulin under

transcriptional regulation has been repeatedly shown to provide some glycemic control in diabetic rodents [83, 132]. However, due to the absence of an acute secretory response, it is doubtful that engineered hepatocytes will provide appropriate glycemic regulation in higher animals, without the risk of hypoglycemic episodes. Myoblasts also lack a regulated secretion pathway, but relative to hepatocytes they offer the advantage of easier retrievability as a biopsy, capability in amplifying into large cell number, and the ability for differentiation into stable myotubes. Moreover, myoblasts have been engineered to constitutively secrete a number of therapeutic proteins, such as ciliary neurotrophic factor [88], erythropoietin [89], human factor IX [90, 91], and human growth hormone [93]. Prior to implantation, myoblasts can be differentiated into myotubes after encapsulation inside polyethersulfone hollow fibers [88, 89], or they can be tissue-engineered *in vitro* into a bioartificial muscle [93]. Both approaches have yielded promising *in vivo* results demonstrating the efficacy of recombinant myoblasts as a living protein delivery device.

In this work, human HepG2 hepatomas and murine C2C12 myoblasts were stably transfected with a human preproinsulin cDNA mutated at the B/C and C/A junctions, so that the ubiquitous endoprotease furin could process proinsulin into an insulin-like molecule. Additionally, the preproinsulin cDNA contained a mutation at the B10 site to enhance insulin stability [72]. For ease of handling, cells were encapsulated in alginate matrix. Histology sections of beads at different time points and insulin-secretion tests showed that encapsulation did not compromise the function of HepG2 and C2C12 cells to an extent that would be incompatible with the objectives of this study.

Besides cells, a second important component of the hybrid construct is the glucose-responsive material. Such types of material have been used in developing

glucose sensors and in controlling the release rate of insulin from a drug delivery device. However, in the latter type of device, loss of insulin activity with time and the need to periodically refill the reservoir are problematic. Insulin-secreting cells result in a continuously refilled reservoir, thus alleviating the problems mentioned above. Various types of glucose-responsive material have been proposed [28, 134, 144]. For use in a hybrid device with cells, an ideal glucose-responsive material should exhibit the following characteristics: it should function under physiologic conditions, including pH, temperature, and physiologically relevant glucose concentrations; release no toxic compounds harming the cells; function with the form of insulin released by the cells, i.e., does not require modified insulin; exhibit high glucose specificity, rapid kinetics, and long-term stability. Although far from ideal, the con A-based glucose-responsive material meets some of the above requirements, including the ability to function with the insulin secreted by cells at physiologic pH and temperature.

Con A is a lectin, which forms a tetramer under physiologic conditions and has four binding sites toward free glucose or glucose residues in a polysaccharide chain. When con A is mixed with polysaccharides, it acts as a crosslinker forming a highly viscous gel. When exposed to a high glucose solution, the gel dissociates into a sol due to the competitive binding reaction between free glucose and the glucose monomers in a polysaccharide chain toward con A. Based on previously reported diffusion chamber experiments [155], the time required for a 2 mm thick material to complete the phase changes is of the order of 30 minutes. It is possible to shorten this response time by reducing the thickness of the material. The glucose concentration of 220 mM used to induce material transformation is well beyond physiologic conditions. The

responsiveness of the material can be brought towards the physiologic domain by using modified con A or other types of polysaccharides [157]. Additionally, factors in fetal bovine serum apparently caused degradation of the con A-based material. All these constitute areas in which material improvements are needed prior to *in vivo* experiments.

Among the insulin-secreting cells used in these experiments, HepG2 cells appeared to be less tolerant to the unfavorable conditions inside the construct that might include hypoxia, and possibly high insulin concentrations in the cell compartment. It is unclear whether the recombinant cells in this study were subject to insulin feedback inhibition. Nonetheless, such inhibition might offer the advantage of preventing excessive insulin accumulation inside the construct. The lower cell viability in experimental vs. control constructs is not surprising, as leaked con A could have a toxic effect towards cells. Tethering con A to the polysaccharide to prevent leakage is thus another modification that may improve the functionality of the construct, especially in the long-term.

Insulin secretion needs to be tightly regulated by glucose to achieve normoglycemia in higher animals and, eventually, humans. In cell-based therapies, potentially autologous non-β cells engineered for insulin secretion are promising, as they are easily available by biopsy and do not entail immune acceptance problems. Although such cells are easily engineered for insulin secretion, introducing appropriate glucose responsiveness is a daunting task. Results presented in this paper indicate that it is indeed feasible to associate constitutively secreting cells with a con A-glycogen glucose-responsive material to generate a hybrid construct with closer to physiologic insulin secretion dynamics compared to cells by themselves or encapsulated in an inert material. Although the con A-glycogen material is adequate for proof-of-concept

studies, modifications are needed to improve the kinetics of sol-gel transformation, the glucose sensitivity, and the long-term stability of the material prior to *in vivo* experiments.

CHAPTER 6

TISSUE ENGINEERED PANCREATIC CONSTRUCT BASED ON PEG-CON A GLUCOSE-RESPONSIVE MATERIAL AND INSULIN-SECRETING CELLS

6.1 Abstract

The proposed cell-material hybrid construct consisted of glucose-responsive material and insulin-secreting cells exhibited insulin-release in response to glucose step changes in the surrounding environment. However, the glucose sensitivity of the con A-glycogen material we previously used was far beyond physiologic conditions. In this study, we explored the potential of improving the glucose-sensitivity of con A-based material through the use of PEGylated con A molecules. Studies showed that PEGylated con A molecules retained the ability in forming glucose-responsive material with glycogen, moreover, the resulting material has improved glucose sensitivity relative to the material formed by unmodified con A molecules. Incorporation of the improved con A-based material into our cell-material hybrid construct, which contained constitutively insulin-secreting myoblasts as the insulin source, the construct released insulin in response to glucose step changes closer to physiologic range.

6.2 Introduction

A major concern in developing a cell-based therapy for treating insulin-dependent diabetes (IDD) is the cells being used, which have to be immune acceptable at clinically relevant amounts, and have to secrete insulin in response to glucose step-changes with kinetics similar to those of β cells. Cells of non-β origin, such as liver [84, 85] and

muscle cells [7], can be retrieved from a patient and genetically engineered *ex vivo* to become insulin-secreting cells. These autologous cells are accepted immunologically and relax the cell availability concerns. However, as β cells regulate insulin secretion in a sophisticated mechanism from gene transcription, translation, to the secretory pathway, the task of engineering non-β cells merely at the gene level becomes very challenging in having cells exhibiting proper secretion kinetics [86].

Instead of relying on cellular and molecular tools, we earlier proposed the first prototype of a cell-material hybrid construct, in which the release of insulin secreted by the cells was controlled by the phase changes of a material barrier composed of glucose-responsive material [155]. Among different types of glucose-responsive material developed for the glucose sensor and insulin delivery [21, 26, 36], we have chosen a concanavalin A (con A)-based material in our proof-of-concept studies. In that study, we were able to change the continuous insulin-secreting characteristic of the cells to glucose-responsive insulin release from the device (Chapter 5). However, there exist problems with the con A-based material that need to be addressed prior to any *in vivo* tests, in particular, the glucose sensitivity of the con A-glycogen material used, which was far from the physiologic range.

Con A is a lectin, which exists as tetramer with four glucose-binding sites under physiological conditions [32]. When mixed with polysaccharides containing pendent glucose molecules, con A acts as a crosslinker to the polysaccharides resulting in the formation of a viscous gel. Exposing the gel to a glucose solution, a competitive-binding reaction between free glucose and glucose on polysaccharides chains toward con A binding sites occurrs. If most of the con A binding sites are occupied by

free glucose, the gel dissociates into a sol [36, 134, 157]. Due to the multivalence effects [158], con A molecules tend to have higher binding affinity towards polysaccharides than free glucose, hence, the glucose concentration required to break down the gel structure is usually very high. Potential solutions in improving the glucose sensitivity of con A-based material includes using a synthetic polysaccharide that has lower con A binding affinity, or modifying the con A molecules so as to have a higher binding affinity toward free glucose and a lower binding affinity toward polysaccharides.

Park et al [8] have shown that by PEGylating the con A molecule, one can increase its binding affinity towards free glucose up to 4-fold. Moreover, due to steric hindrance, it is also expected that PEGylated con A would have lower binding affinity towards polysaccharides. In addition, PEGylation is a tool widely used in improving the biocompatibility and stability of proteins [159, 160], so using PEGylated con A could be advantageous in reducing the immunogenicity of the resulting gel. In this study, we demonstrated that the glucose sensitivity of con A-based material can be improved by using PEGylated con A molecules. Experiments with the cell-material hybrid construct containing the improved material and continuous insulin-secreting myoblasts were also performed, which provided further proof that it is indeed feasible to develop a tissue-engineered pancreatic substitute combining the genetically engineered cells and the glucose-responsive material. The significance of this approach and future directions are discussed.

6.3 Materials and Methods

6.3.1 Con A PEGylation and preparation of glucose-responsive material

Con A was modified with PEG using Acrl-PEG-NHS (Molecular weight of 3,400, Nektar Therapeutic, Huntsville, AL), which has two functional groups: the NHS group (N-hydroxylsuccinimide) is reactive towards lysine groups and is widely used for protein PEGylation; The Acrl (acrylate) group offers the possibility of vinyl polymerization, which can be used to tether con A to polymer chains, if needed. Such tethering was not implemented in the present study. Con A (Amersham Biosciences, Piscatway, NJ) was first dissolved in PBS at 15% (w/v). Weighted Acrl-PEG-NHS was then slowly added to the con A solution at molar ratios of Acrl-PEG-NHS to con A of 5.0:1.0 and 2.5:1.0. The reaction was carried out in an ice bath for 30 minutes. PEGylated con A tended to form white precipitates and compromised its ability to form glucose-responsive material with glycogen when left too long in the solution. Hence, glucose-responsive materials were prepared immediately after the reaction. The preparation of con A /PEG-con A-glycogen was based on the published protocol by Taylor et al. Briefly, a con A solution with or without the step of PEGylation was mixed with a 30% glycogen (Sigma) solution, which was prepared by dissolving glycogen in PBS at 60% w/v followed by mixing with a 0.2 M $NaIO_4$ (Sigma) solution at 1:1 volume ratio for 24 hours in the dark. This step converted the glycogen into the aldehydic form that would later react with the lysine groups on a con A molecules into a Schiff Base. The viscous gel was formed immediately after stirring the mixtures of glycogen and con A and was left at room temperature 24 hours followed by 1 hour immersion in 1 mg/ml $NaBH_4$ (Sigma) at 4°C, which stabilized the Schiff Base. Finally, the gel was washed at least three times with

PBS buffer and stored in PBS at 4°C for later experiments.

6.3.2 Glucose-responsiveness and leakage experiments

Con A-based materials were cut into pieces of similar size and weight and loaded into tissue culture inserts with membranes of 0.02-micron pore size (Nalge Nunc International, Rochester, NY) with 0.5 ml of PBS. After putting each insert in a well of a 24-well plate, 0.5 ml PBS with different glucose concentrations (440, 220, 110, 55, 10, and 0 mM) was added to the well outside the inserts. The final glucose concentrations in the wells were 220, 110, 55, 27.5 5, and 0 mM after equilibrium because equal volume of glucose-free PBS and glucose solution were added on the inside and outside side of the insert. Pictures of gels were taken before and 30 minutes after the glucose solution was added. After 6 hours of incubation, samples of solution were taken from each well outside the insert and analyzed for protein content by Coommassie Blue assay (Pierce, Rockford, IL). Protein standards were constructed on the basis of known concentrations of con A.

6.3.3 Cell culture and encapsulation

Stable, continuous insulin-secreting C2C12 mouse skeletal myoblasts were developed in a previous study reported in Chapter 5. Cells were cultured as myoblasts in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Herndon, VA) supplemented with 10% Fetal bovine serum (Hyclone, Logan, UT), 1% penicillin/streptomycin (Mediatech), and 1 μg/ml puromycin. To facilitate cell loading into hybrid constructs, C2C12 cells were encapsulated in 2% alginate (ISP Inc., San

Diego, CA) at a density of $7x10^7$ cells/ml of alginate following the general protocol published by Stabler et al [71], except that a poly-L-lysine membrane and a final alginate coating were not applied.

6.3.4 Construct Experiments

To test the glucose-responsiveness of con A-based materials and to run cell-material hybrid construct experiments, a previously designed cell-material hybrid device with the geometry shown in Figure 5.2 was used but the constructs used in cell-free experiments are in smaller dimensions (Figure 6.1). Briefly, two polycarbonate plates (3 mm in thickness), each with a hole in the center, constituted the main structural components of the construct. Two silicon sheets (2 mm in thickness), also each with a hole in the center, were used to separate the membranes that enclosed the glucose-responsive material and the insulin source. The material compartment was sandwiched between two insulin-permeable membranes, and the other compartment was sequestered on the other side by an insulin-impermeable membrane.

For cell-free experiments, two 0.1 μm pore size polycarbonate membranes (Whatman Inc.) and a cellulose ester membrane of 6 kDa molecular weight cut off (Spectrum Lab, Rancho Dominguez, CA) were used. Pieces of con A-based material of approximately 0.8 ml total volume were transferred by a spatula and used to fill the hole of the silicon sheet on top of a polycarbonate membrane. Then, the other polycarbonate membrane was positioned on top of the material to complete the material barrier. The material barrier with another silicon sheet and a cellulose ester membrane were then tightly assembled together by two polycarbonate plates and screws. A 0.8 ml volume of

a 2 mg/ml FITC-insulin solution in PBS was then injected into the insulin compartment. The whole construct was immediately immersed in a beaker containing 20 ml of 0.2 mg/ml BSA (Sigma) in PBS. The beaker and the construct were then placed on top of an orbital shaker that provided adequate mixing. 2 hours after incubation, glucose was directly added to the beaker to increase the glucose concentration. Samples were taken every 20 minutes during experiments, and the same amount of buffer was added back to maintain constant volume inside the beaker. The purpose of cell-free experiment was mainly to characterize the change of insulin permeability of material after exposing to glucose, thus no step down of glucose was performed. At the end of the experiment, fluorescence intensity of each sample was measured by a fluorescence plate reader (Spectra Max Gemini Plate Reader, Molecular Devices Corp., Sunnyvale, CA). The concentrations of FITC-insulin were calculated using a standard curve constructed on the basis of known FITC-insulin concentrations. These experiments were performed at room temperature.

The membranes used in the cell-material hybrid construct were: a polyethersulfone membrane of 3kDa molecular cut off, positioned on the external side of the cell compartment, a 0.02 μm Anodisc membrane (Whatman Inc., Clifton, NJ), positioned between the material and the cells, and a 0.1 μm pore size polycarbonate membrane (Whatman Inc.) positioned between the material and the surrounding medium. Membranes used in cell-free experiments were different from the ones used in cell-material hybrid construct experiments because the latter tend to breakdown when assembled within the cell-free construct, which has smaller dimensions than the cell-material hybrid construct. The experimental construct containing con A-based

material barrier was assembled as previously described, except approximately 2 ml of material was used. For control experiments, the con A-based material was replaced with calcium alginate. In these constructs, the material compartment was first assembled with the Anodisc and polycarbonate membranes and the polycarbonate plates sandwiching the silicon sheet tightly. Sodium alginate (ISP Inc.) filter-sterilized through 0.2 μm syringe filter (PALL Life Sciences), was then injected, filling the space in between the membranes. The whole construct was immersed in a 1.1% $CaCl_2$ solution for 30 minutes to ensure gelation of the alginate inside the membrane sandwich.

To assemble the cell compartment, 1 ml of alginate-encapsulated C2C12 cells were first placed on the top of the 3 kDa MWCO polyethersulfone membrane on the lower polycarbonate plate. The material barrier was then laid on top of the cell compartment, followed by another polycarbonate plate laid on top of the compartments, and the whole construct was tightly assembled by screws. Approximately 1 ml of the culture medium was then injected into the cell compartment. The whole construct was immediately transferred into a beaker containing 50 ml of culture medium with 5 mM glucose supplemented 2.5% horse serum. The beaker and the construct were then placed inside a tissue culture incubator on top of an orbital shaker that provided adequate mixing.

To allow insulin to accumulate inside the cell compartment, each construct was cultured for 20 hours prior to glucose concentration changes. At the end of this incubation period, the medium was replaced with fresh culture medium, so when the experiment started at t=0, the surrounding medium was insulin-free and contained 5 mM glucose. For the glucose step up, 0.225 g of glucose was directly added to the beaker to increase the glucose concentration to 30 mM. For the glucose step down, the medium

was completely replaced with fresh culture medium with 5 mM glucose. Two square-waves of glucose were implemented to test the glucose-responsiveness of the hybrid construct. Samples were collected every 30 minutes during experiments, and the same amount of culture medium was added back to maintain constant volume inside the beaker. All samples were stored at -20^{o}C for later insulin assay. At the end of the experiment, the constructs were disassembled, and the alginate-encapsulated cells were collected to assess cell viability.

6.3.5 Analytical Techniques

To verify that Acrl-PEG-NHS molecules indeed conjugated to con A molecules, the fluorescamine (Sigma) assay [161] was used. Fluorescamine reacts with primary amines such as lysine to form a fluorescent product. As more PEG molecules attached to a con A molecule through the reaction between NHS and lysine groups, fewer primary amine groups were available for fluorescamine. Immediately after PEGylation of con A, the PEgylated con A solution was diluted with PBS into different concentrations ranging between 15-300 μg/ml. After dilution, 150 μl aliquots of samples were pipetted into the wells of 96-well plates, and 50 μl of 3 mg/ml fluorescamine dissolved in acetone was then added to each well. The fluorescence was determined by a fluorescence plate reader (Spectra Max Gemini Plate Reader, Molecular Devices Corp., Sunnyvale, CA). Fluorescence intensity was later plotted as a function of concentration. Slopes were obtained for both modified and unmodified con A samples. The slop ratio of PEG-con A to unmodified con A was calculated and prorated to the number of primary amine groups (12 lysine groups plus one terminal amine group) in Con A to determine the extent of

modification.

6.3.6 Cell viability and insulin assay

To measure the viability of alginate-encapsulated cells, beads were dissolved in a 2.2% sodium citrate solution, and the resulting cell suspension was centrifuged for 3 minutes at 110g. Pelleted cells were re-suspended in culture medium and mixed with trypan blue (Sigma) at 1:1 volume ratio. Cell viability in the final suspension was determined on a hemocytometer under a microscope by trypan blue exclusion. Insulin in cell-material hybrid construct experiments was assayed by a human-specific insulin radioimmunoassay (Linco Research, St. Charles, MO).

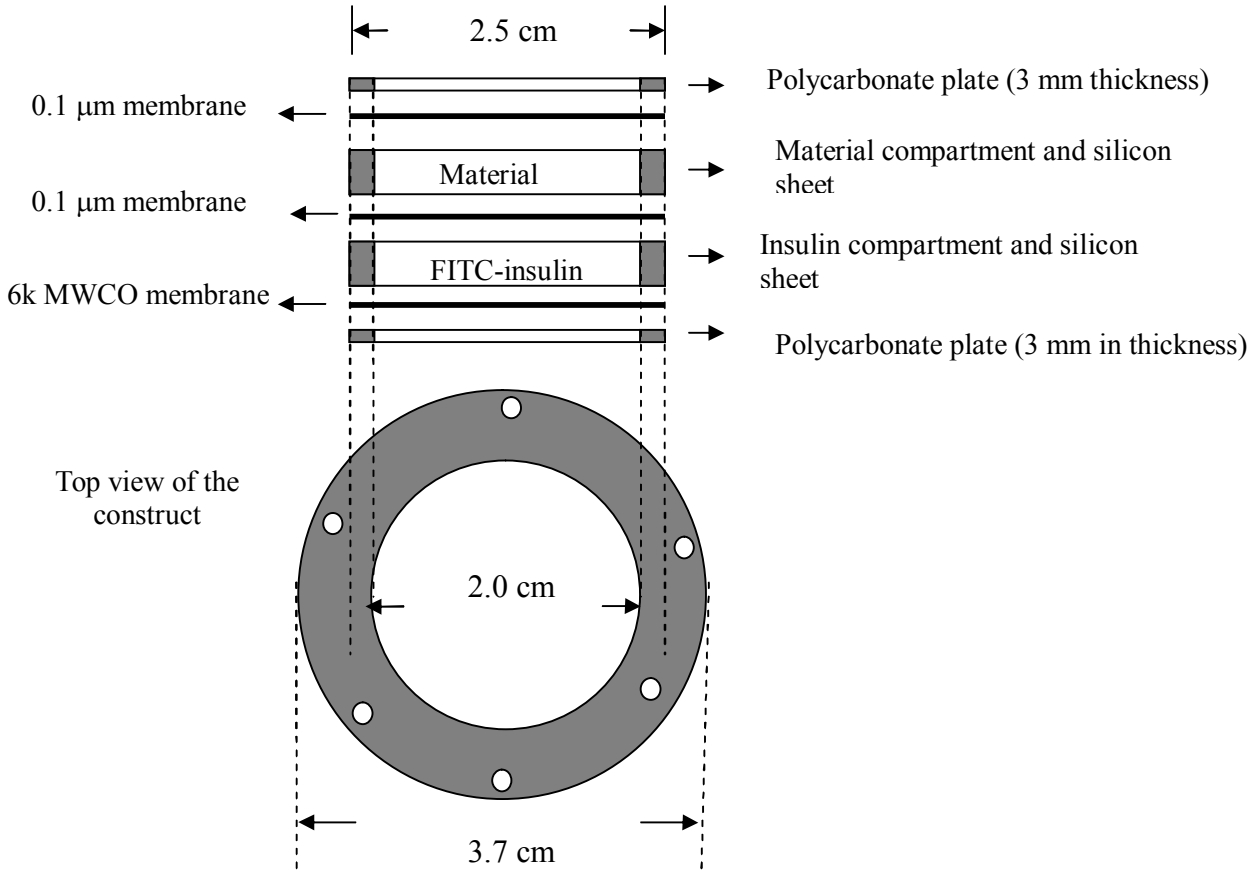


Figure 6.1 Configuration of constructs used in cell-free experiments. The con A-glycogen material was placed in the 2.0 cm diameter hole of a 2 mm-thick silicon sheet and was sandwiched between two 0.1 μm pore-size polycarbonate membrane. The insulin compartment consisted of 2 mg/ml FITC-insulin solution positioned in the 2.0 cm diameter hole of a 2 mm-thick silicon sheet and bounded on one side by the material compartment and on the other side by a 6k Dalton MWCO cellulose ester membrane. Polycarbonate plates with 6 screws were used to fix everything in place. The figure is not drawn to scale.

6.4 Results

PEGylation of Con A molecules was verified by the fluorescamine assay and a typical result is shown in Figure 6.2. The slope ratio of PEG: con A (molar ratio 5) to unmodified con A is 62.7±9.2%, which indicated that an average of four to five lysine groups per con A molecule were reacted with PEG molecules after the reaction (13 primary amine groups per con A molecule x(1-62.7%) =4.8). Similar calculation was done with PEG: con A (molar ratio 2.5) and a slope ratio of 82.7±3.1 was obtained, which indicated that about two to three PEG molecules is attached to one con A molecules (2.2). The notation of "PEG 2.5-Con A" and "PEG 5-con A" will be used for PEG/con A molar ratio of 2.5 and 5, respectively.

Shortly after mixing the glycogen and PEG-con A solutions, a viscous gel was formed. The gel formed with PEGylated con A was softer and less opaque compared to the gel formed with unmodified con A. Within 30 minutes after exposing the gels to different glucose concentrations, the PEG 5-con A-glycogen material remained as gel at 5 mM glucose but turned into a sol at 27.5 mM glucose, whereas the PEG 2.5-con A-glycogen material was partially converted to sol at 55 mM glucose and completely converted to sol at 110 mM. Most of the unmodified con A-glycogen material remained in the gel state at glucose concentrations tested (Figure 6.3). These results thus indicated that the glucose-responsiveness of the con A-based material can be improved by using PEGylated con A. PEGylation of con A is expected to increase the molecular weight of con A molecules, and hence decrease its permeability through membranes. However, 6 hours after exposing the gel to different glucose concentrations, higher protein concentrations were detected outside the 0.02-μm pore size tissue culture inserts at 0-55

mM glucose when PEGylated con A was used relative to unmodified con A (Figure 6.3).

Experiments with FITC-labeled insulin were carried out at room temperature and 20 ml PBS with 0.2 mg/ml BSA as the surrounding solution. The construct was incubated in glucose-free PBS for 2 hours, during which time the material was in the gel state. Then, glucose were added into the beaker to increase the glucose concentration and the experiments were continued for another 2 hours. The FITC-insulin release rates during glucose-free and high-glucose periods were calculated by the slopes of the least squares fit of the released FITC-insulin concentration vs. time data; rates at high glucose periods were normalized to the corresponding glucose-free release rate during the 0-2 hour period. The results from three independent experiments were shown in Figure 6.4. The change of FITC-insulin release rates from 0 to 27 mM glucose was most pronounced with PEG 5-con A-glycogen material, indicating that material formed with PEG 5-con A-glycogen can be used to control insulin release at glucose concentrations close to the physiologic range.

As PEG5-con A-glycogen exhibited glucose responsiveness closest to the physiologic range, it was the material used in construct experiments with cells. Figure 6.5 (A, B and C) show a typical insulin-release profile from the cell-material hybrid construct containing three different types of materials: PEG5-con A-glycogen, alginate, and con A-glycogen, under glucose square-wave changes from 5 to 30 to 5 mM. The average insulin release rate in each glucose concentration was calculated from the slope of the least squares fit of the released insulin concentration vs. time data; each rate was normalized to the corresponding low-glucose release rate during the 0-2 hour period, which was set equal to 1. The results from three independent experiments are shown in

Figure 6.6. Statistical differences were evaluated by the Student's t-test and were considered significant at $p<0.05$. Constructs containing alginate or unmodified con A-glycogen material acted as continuously insulin-release devices, as they did not respond to changes in the surrounding glucose concentration. On the other hand, insulin release rates from the construct containing PEG 5-con A-glycogen changed with the implemented step changes of glucose concentration: the rates during high glucose were, on average, 2- to 3- fold higher than those during the low glucose periods. However, we also noticed that the PEG 5-con A-based material became more fluid after 32 hours of experiment.

At the end of 32 hour-long incubations, cell viabilities from constructs containing different types of material barriers were evaluated by trypan blue and are shown in Figure 6.7. When constructs were exposed to 5-30 mM glucose, only 20% of cells remained viable after 32 hours in all types of construct. Viabilities were significantly higher in constructs containing alginate or con A-glycogen and cycled between 25 and 220 mM glucose (Data from Chapter 5).

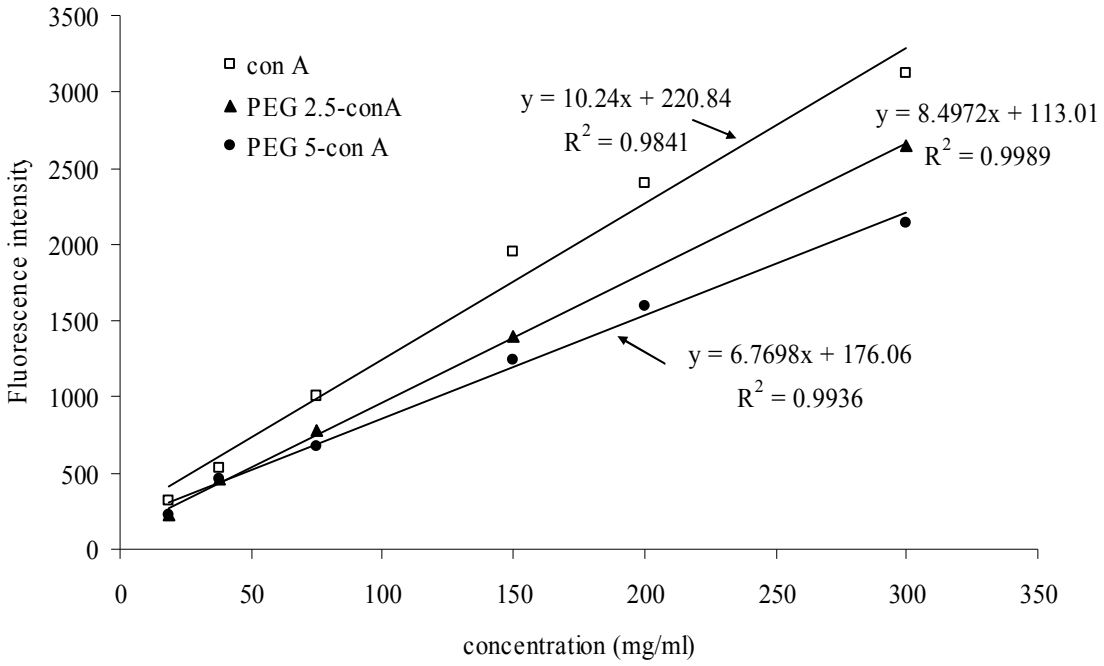


Figure 6.2 Linear regression analysis of fluorescamine standard curves for nature and PEG-modified con A.

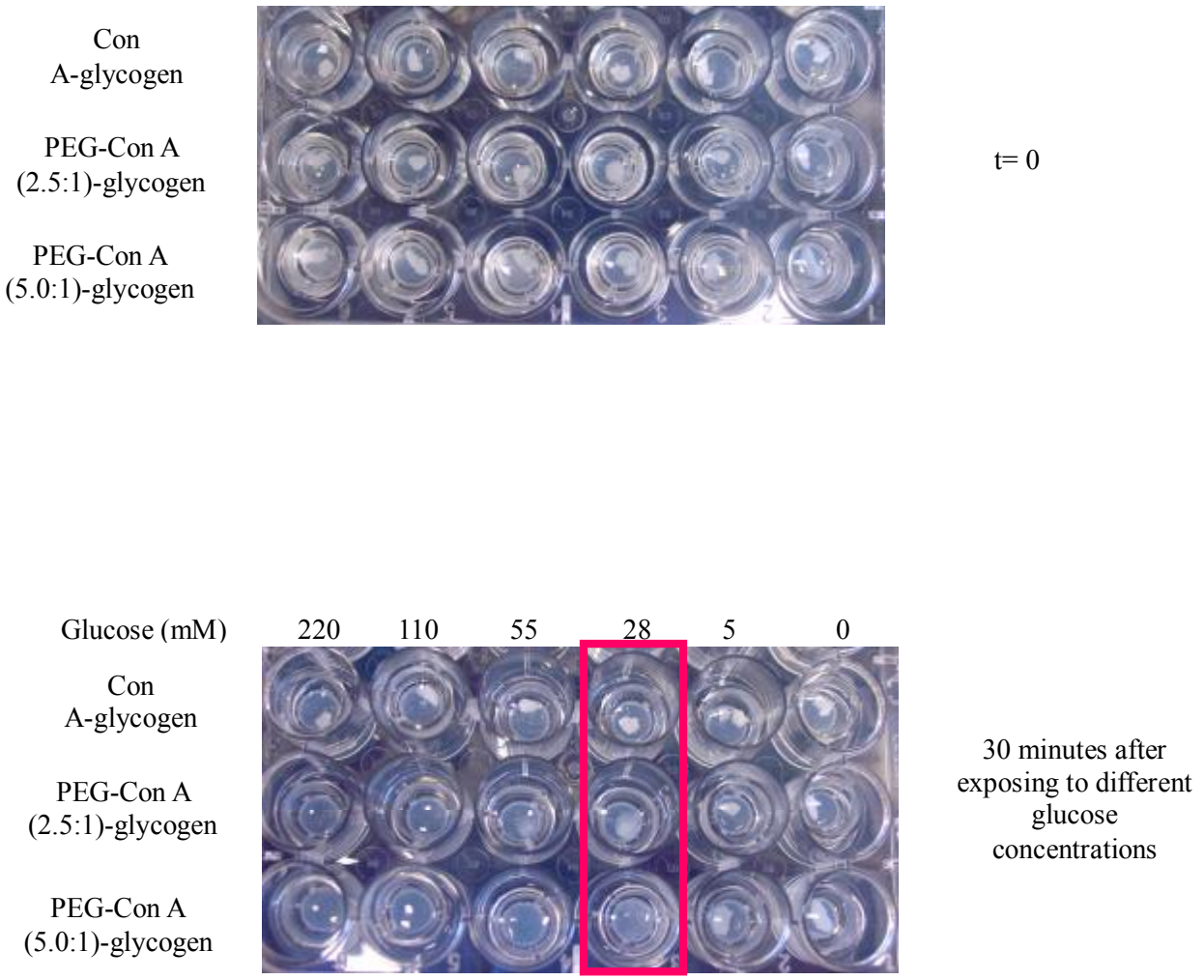


Figure 6.3 Con A-based glucose-responsive materials before (top) and 30 minutes after exposing to glucose solutions (bottom).

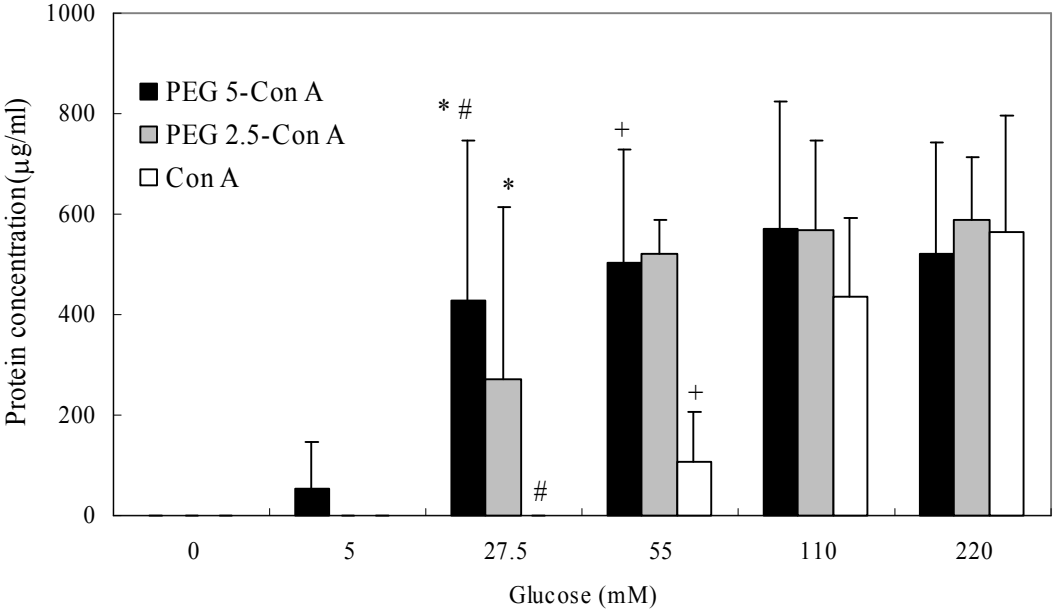


Figure 6.4 Amount of con A molecules passed through 0.02-μm pore size membrane tissue-culture inserts 6 hours after exposing to glucose solutions (n=3). # * and + indicate pair-wise statistical comparisons using a one-tailed t-test, $p<0.05$. Error bars indicate standard deviation.

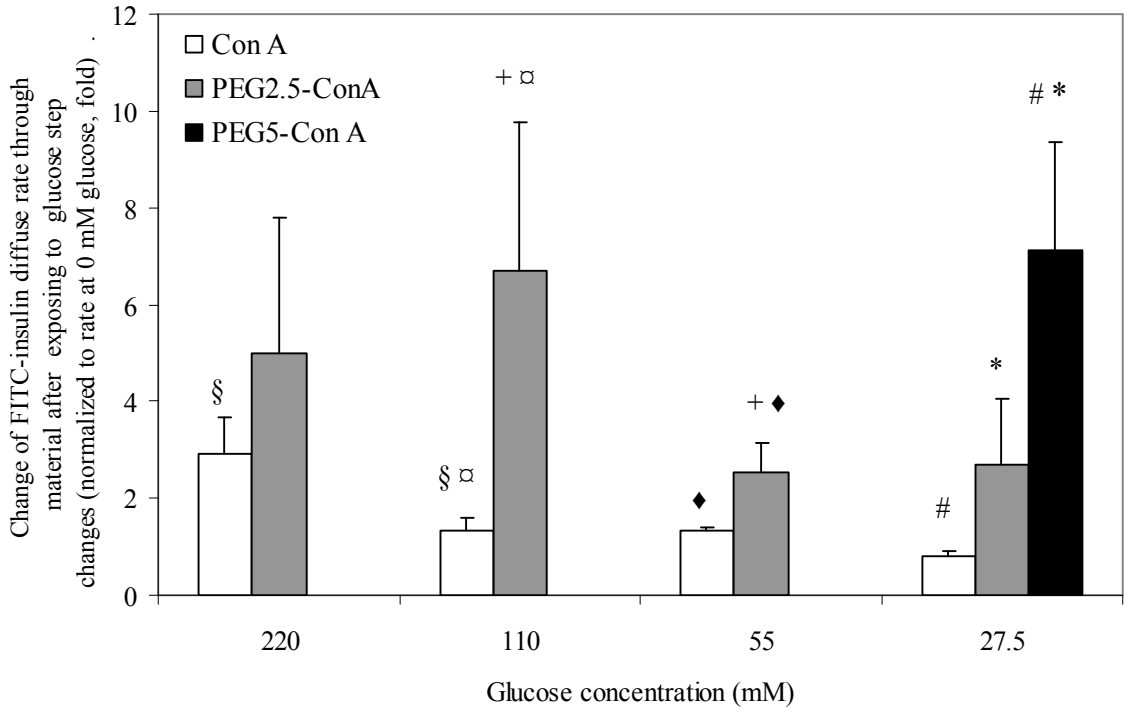


Figure 6.5 FITC-insulin release rates from cell-free constructs containing different types of con A-based materials in different glucose concentrations relative to those in glucose-free solutions (materials at gel state) (n=3). # *§ ¤ and ♦ indicate pair-wise statistical comparisons using a one-tailed t-test, $p<0.03$. Error bars indicate standard deviation.

(A)

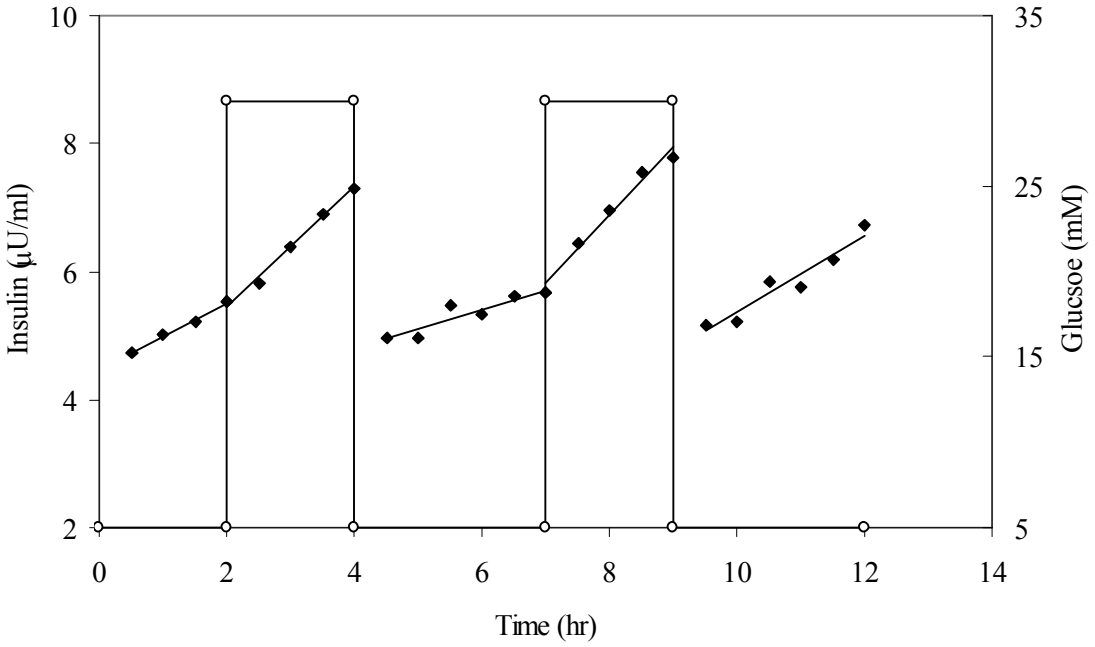
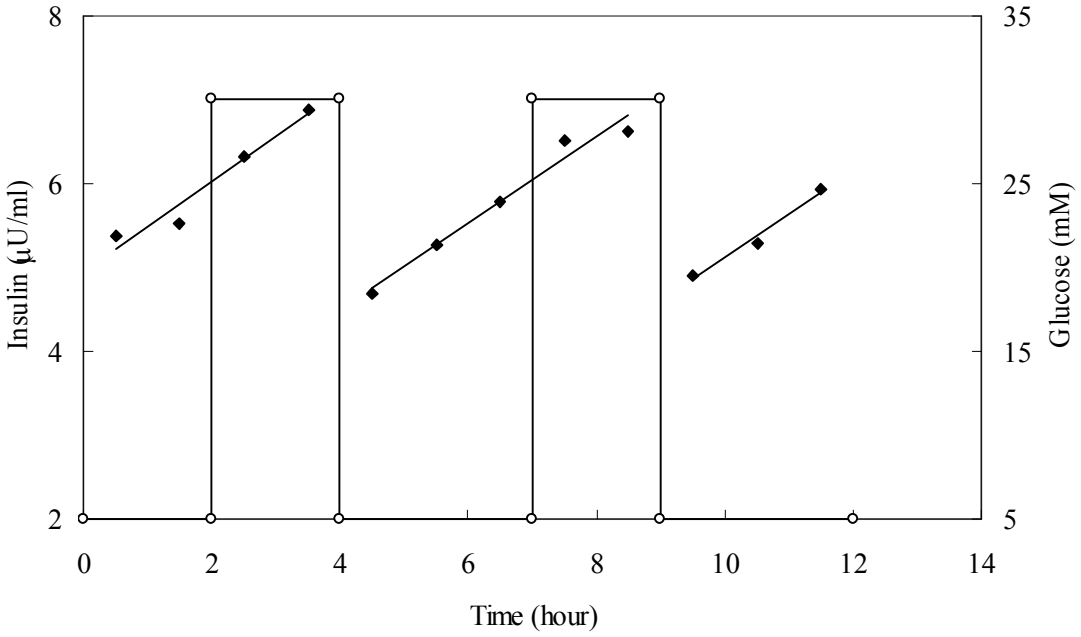


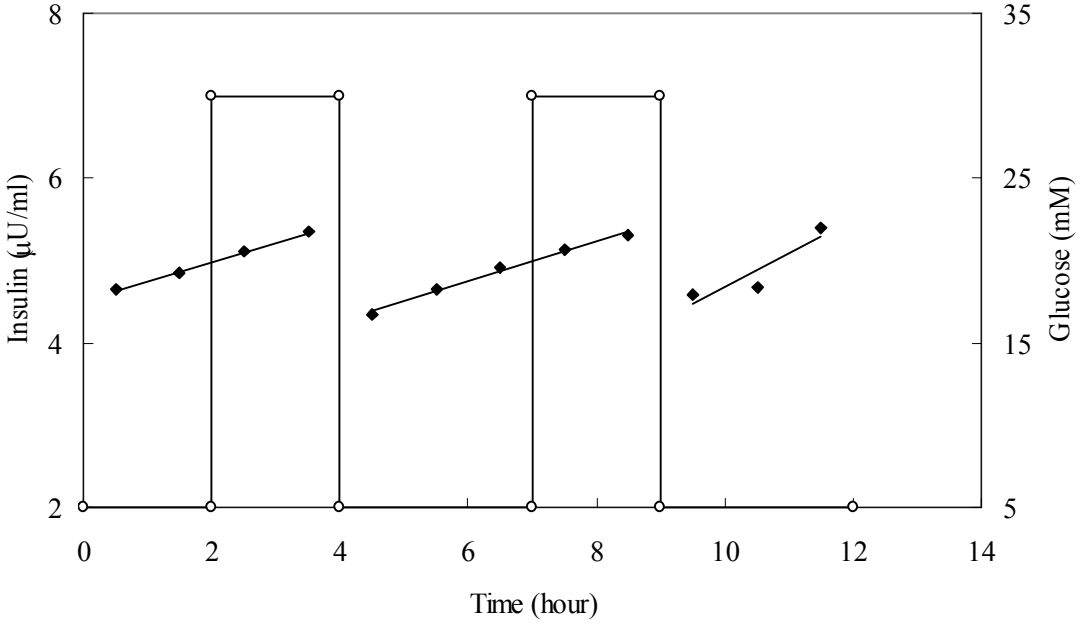
Figure 6.6 Typical insulin-release profiles from hybrid constructs of alginate-encapsulated insulin-secreting C2C12 cells with PEG 5-con A-glycogen (A), alginate (B), and con A-glycogen (C).

Figure 6.6 (continued).

(B)



(C)



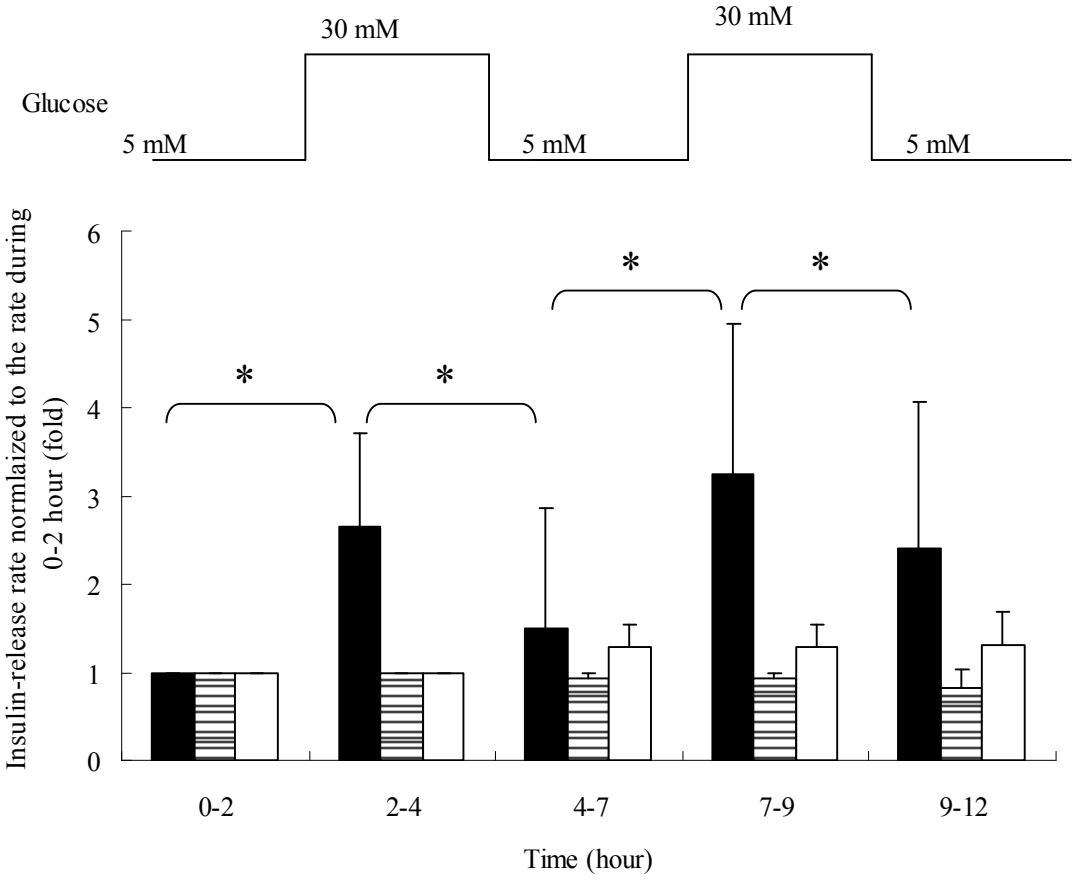


Figure 6.7 Insulin-release profiles from hybrid constructs of alginate-encapsulated insulin-secreting C2C12 cells with PEG 5-con A-glycogen (filled bar), alginate (dashed bar), and con A-glycogen (blank bar). In each independent experiments, insulin release rates were normalized to the rate during 0-2 hours, which was set equal to 1 (n=3). * indicate pair-wise statistical comparisons using a one-tailed t-test, $p<0.05$. Error bars indicate standard deviation.

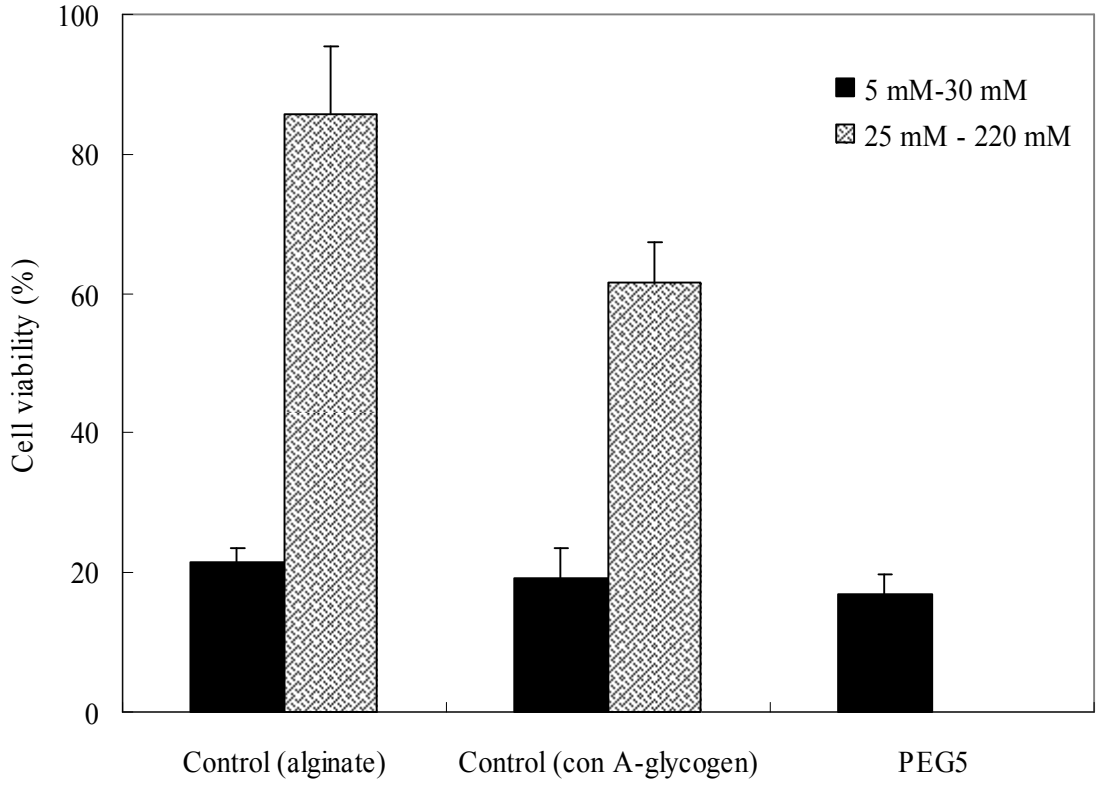


Figure 6.8 Viabilities of alginate-encapsulated C2C12 cells at the end of construct experiments (n=3). Filled bars are experiments performed under 5-30 mM glucose square-wave changes; Dashed bars are data obtained from the previous experiments under the same experimental set up except the glucose square-wave changes were between 25-220 mM (n=3).

6.5 Discussion

Previously, we developed a cell-material hybrid pancreatic construct in which the kinetics of insulin release are dominated by the phase changes of glucose-responsive material. Cells inside the construct can be different types of non-β cells retrieved from a patient and engineered for insulin expression, and they act as continuous, unregulated insulin secretors. As the cells are of autologous origin, they relax the immune-acceptance problems intrinsic to the use of xeno- or allogeneic cells. Since the release of insulin from the construct is mainly controlled at the material level, the functionality of the glucose-responsive material, including the glucose sensitivity, kinetics, and reversibility of phase changes, are keys to the development of such construct. We have chosen a con A-based glucose-responsive material in our proof-of-concept experiments because it has high specificity towards glucose, shorter response time relative to other types of glucose-responsive hydrogels, and it functions with insulin secreted by the cells. However, several issues remained with the con A-glycogen material that need to be addressed, in particular, bringing the glucose sensitivity of the gel towards physiologically relevant glucose concentrations.

When exposing a con A-glycogen hydrogel to a glucose solution, the binding states of the con A molecules can be described in the following equations:

[con A] + [glycogen] $\leftrightarrow$ [con A:glycogen]

[con A] + [glucose] $\leftrightarrow$ [con A:glucose]

Binding constant of $K_{con\ A:glycogen} = \dfrac{[con\ A : glycogen]}{[con\ A][glycogen]}$

$$[\text{con A:glycogen}] = K_{con\ A:glycogen}[\text{con A}][\text{glycogen}] \qquad (1)$$

Binding constant of $K_{con\,A:glucose} = \frac{[con\,A : glucose]}{[con\,A][glucose]}$

[con A: glucose]=$K_{con\,A:glucose}$ [con A][glucose] (2)

Dividing equation (1) by (2):

$$\frac{[con\,A:glucose]}{[con\,A:glycogen]} = \frac{K_{con\,A:glucose}\,[glucose]}{K_{con\,A:glycogen}\,[glycogen]} \quad (3)$$

To have the material in the sol state, most of the con A molecules have to bind to the free glucose, in other words, [con A:glucose] has to be larger than [con A: glycogen]. As shown in equation (3), the situation can be achieved by increasing the concentration of free glucose. However, due to multivalency effects, con A generally has higher affinity towards polysaccharides than towards free glucose (e.g. $K_{conA:dextran}$=1.5x10^4 M^{-1} and $K_{conA:glucose}$=320 M^{-1} [162]), hence, the glucose concentration required to induce the con A-glycogen gel into a sol is high beyond physiological range. One potential solution in improving the glucose sensitivity of the con A-based glucose-responsive material is by adjusting the binding constants, more specifically, decreasing the $K_{conA:glycogen}$ and increasing $K_{con\,A:\,glucose}$.

Inspired by the results reported by Park et al [8], which indicated that PEGyled con A molecules increased their binding affinity towards free glucose and possibly reduced their binding affinity towards polysaccharides through steric hindrances, we hypothesized that the glucose responsiveness of developed con A-glycogen system can be improved by using PEGylated con A molecules. Results were quite promising in terms of improving the sensitivity of con A-glycogen material: the hydrogel formed by PEG 5-con A-glycogen turned into a sol at a glucose concentration as low as 28 mM. In addition, using PEG 5-con A based material in our proposed cell-material hybrid construct, we

were able to convert the constitutively insulin-secreting characteristics of genetically engineered myoblasts into a glucose-responsive insulin release from the device under the square -wave changes of glucose concentrations between 5 mM and 30 mM.

The con A molecule has twelve lysine groups and only one lysine group is located within the saccharide-binding site of con A. Although different studies have reported that modifying con A molecules through lysine groups does not compromise the glucose-binding properties of con A molecules [163, 164], little is known as to which lysine group among the twelve or number of lysine groups being modified has the most impact on the functionality of con A molecules. For instance, there is evidence that acetylation of con A molecules through lysine residues leads to the disruption of salt links between 114 and 116 lysine groups, and consequently, dimeric instead of tetrameric con A molecules were formed at pH 7 [32, 165]. It is known that the binding affinity of dimeric con A towards polysaccharides is lower than that of tetrameric con A [166, 167]. As the conjugation of PEG molecules via covalent attachment to the lysine groups of con A is not a site-specific reaction, the random PEGylation of Con A molecules could result in different types of con A molecules, including molecules that have higher binding affinity toward free glucose as shown by Park et al [8]; molecules that have lower binding affinity toward polysaccharides due to steric hindrance of PEG molecules; and dimeric instead of tetrameric con A molecules at neutral pH, which are known to have lower binding affinity toward polysaccharides. Clearly, more experiments are needed to further understand the exact underlying cause(s) of the improved glucose sensitivity of the material.

Based on the above discussion, PEGylation of con A could lead to an improvement

of glucose sensitivity of the resulting gel but also cause con A molecules to be easily dissociated from the gel structure under low glucose concentration. This provides the possible explanation that even though the PEGylation is expected to increase the molecular weight of con A and hence decrease its permeability through porous membranes, results from the leakage experiments showed that some con A molecules leaked out the system at glucose concentration as low as 27.5 mM. Although Ueno et al [168] showed that the PEGylated con A has reduced immunogenicity, leak of PEGylated con A in the body is still possible to induce immune response. In addition, as con A is the key element in retaining the glucose responsiveness of the hydrogel, loss of con A will compromise the stability of the material. Hence, tethering PEGylated con A to the polysaccharides constitutes a critical issue in improving both the biocompatibility and stability of the material.

The fold differences of insulin-release rates between high and low glucose concentrations between cell-free (7-fold) and cell-material hybrid construct (3-fold), can be attributed from differences in experimental conditions: FITC-insulin vs. insulin secreted by the cells, surrounding solutions, temperature, and whether there was a 20 hours of incubation time in 5 mM culture medium prior to glucose-square wave changes.

Another notable finding was the significant loss of cell viability at the end of the 32 hour-long experiments, which we did not observe with previous experiments performed under high glucose square-wave changes. Since there was no significant difference between the control (both alginate and con A-glycogen) and experimental constructs (PEG 5-con A-glycogen), we speculate the loss of cell viability to be due to the unfavorable conditions of hypoglycemia inside the constructs. Modifications aimed at

improving the construct design such as enhancing the nutrient-transport by increasing the surface to volume ratio are described in Chapter 7 of this thesis.

Smart hydrogels that undergo phase transition in response to specific molecules have many technological applications in sensors, drug delivery devices and actuators. The study presented here showed that it is possible to improve the glucose responsiveness of con A-based material by altering the binding affinity of con A molecules through PEGylation. Moreover, by associating the improved con A-based hydrogel with genetically engineered cells, we further demonstrated the possibility of utilizing the glucose-responsive material as a controlled barrier for the insulin released by the cells.

CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Conclusions

Cell source constitutes a major problem in developing a tissue engineered pancreatic substitute, and one of the major selection criteria is how cells secrete insulin in response to stimuli. In this thesis, we have contributed to this field by characterizing and improving the secretion properties of continuous-β cells and cells of non-β origin. A concern with the use of β-cell lines is that cells continue to grow post-encapsulation, and due to nutrient limitation, the homogenous distribution of the cells within the construct remodel in long-term culture. In Chapter 3, we attempted to understand whether the remodeling process within the alginate-encapsulated βTC cells has any effects on the insulin-secretion profile of the capsules. Studies showed that the secretion profile from the beads indeed changed after long-term culture, and that the changes can be prevented if cell growth was inhibited. Although the clinical relevance of this study is not fully understood, as whether the change of insulin secretion dynamics has any effect on the *in vivo* efficacy of the capsules, the results pointed out that the remodeling process within the construct could leads to the functional changes of the implants, and growth inhibition with continuous β-cell lines appeared to be necessary not only to prevent overgrowth *in vivo* but to prevent any unwanted alterations of secretion dynamics.

In the second part of the thesis, we proposed a novel idea of improving the secretion properties of genetically engineered cells of non-β origin. In this, glucose responsiveness was built into the system at the material level instead of relying on

transcriptional control at cellular level. Con A-based glucose-responsive material was identified as a suitable material for our proof-of-concept experiments. Two of the non-β cells, human HepG2 hepatoma and murine C2C12 myoblasts, were genetically engineered to become constitutively insulin secretors. Loading these cells into the proposed prototype of cell-material hybrid construct, which contained con A-glycogen in the material barrier, the continuous insulin-secretion property from the cells changed into a glucose-responsive, more physiologically relevant, insulin-release device. The advantages of such hybrid construct are as follows: (1) different types of non-β cells can be retrieved from the patient as biopsy thus eliminating the concern of cell availability; (2) cells are of autologous origin and of non-β cell phenotype, which relaxes the immune acceptance problems intrinsic to the use of xeno or allogeneic cells and autoimmunity towards β cells; (3) this approach aims at genetically engineering cells ex vivo, which prevents any potential concerns of *in vivo* gene therapy, such as the immunogenecity of gene-delivery vehicles; (4) the sluggish secretion property of genetically engineered non-β cells is improved by the use of glucose-responsive materials, more physiologic insulin release by the device; (5) the construct offers the advantages of retrievability if needed; (6) cells act as continuous insulin secretor, patients will have better life-styles without the use of needles for insulin-injection or the burden of refilling insulin pumps.

As the con A-glycogen material used in the proof-of-concept experiments undergoes phase changes at glucose concentrations exceeding physiological ranges, the last part of the thesis aimed at improving the glucose sensitivity of con A-based material. Studies showed that the glucose sensitivity of the hydrogel formed by PEG 5-con A and glycogen was closer to physiological ranges than that of the material formed by

unmodified con A, which presented the possibility of developing a glucose-responsive material with proper sensitivity through the modifications of glucose-binding molecules. The hybrid construct containing PEG-5 con A-glycogen and genetically-engineered continuous insulin-secreting C2C12 cells exhibited glucose-responsive insulin release under glucose concentrations closer to physiological ranges, indicating the feasibility of developing a proper tissue engineered pancreatic construct based on glucose-responsive materials and insulin-secreting cells.

7.2 Future directions

7.2.1 Continuous β cell lines

Just as islets from different species exhibit different biphasic insulin-secretion patterns upon glucose stimulation, such as the ones between mouse and rat (mouse islets exhibit a sharp first phase of secretion, with the second phase of insulin release being at a flat and much lower rate than the maximum rate of the first secretion phase; and rat islets have the insulin secretion rate during the second phase comparable to the maximum rate of the first phase [119]), *in vivo* studies are needed to understand whether the subtle changes in insulin-secretion profile from alginate-encapsulated continuous-cell lines with time could result in compromised glycemic regulation in small and, more importantly, large animal models.

Furthermore, as the remodeling processes were the consequences of series events, including the development of nutrient gradients within the beads, accumulation of cell death byproducts, degradation of the supporting matrix, and changes of the cell-cell and cell-matrix interactions from those in freshly encapsulated preparations, identifying the

exact cause of the secretory profile changes with time would be interesting and significant for the use of continuous-cell lines as the insulin source.

7.2.2 Development of cell-material hybrid constructs

The proposed cell-material hybrid construct is the first attempt in combining glucose-responsive materials with insulin-secreting cells for a tissue-engineered pancreatic substitute. Clearly, there remain many issues with the construct that need to be addressed prior to any *in vivo* studies. The following paragraphs discuss possible future directions for the construct development.

7.2.2.1 Improving properties of glucose-responsive material

The key factor in developing the proposed cell-material hybrid construct is the functionality of the glucose-responsive material. The current con A-based material is only suitable for proof-of-concept experiments and needs to be improved prior to any clinical applications.

The first issue is the glucose sensitivity of con A-based material, which needs to be in the range of 5-16 mM glucose. Studies shown in Chapter 6 indeed presented a possibility in improving the glucose-sensitivity of con A-based hydrogel through the PEGylation of con A molecules. However, as the PEG-NHS reacted randomly with the 12 lysine groups on a con A molecule, little is known at the molecular level as to which lysine or the number of lysine groups that need to be modified to obtain a con A molecule with proper binding affinity, both towards free glucose or polysaccharides, for forming a sensitive material. On the other hand, it is known that the con A molecule has different

binding-affinity towards different types of polysaccharides, for example, it has higher affinity towards branched polysaccharides than toward linear polysaccharide chains [169]. Using other types of polysaccharides in the system constitutes another approach in improving the glucose sensitivity of the con A-based hydrogel. Overall, a thorough understanding of glucose-binding molecules in conjunction with the use of different types of polysaccharides constitute promising future approaches for improving the glucose-sensitivity of con A-based materials.

A second issue is how fast the material undergoes phase changes. The response time of 2 mm thick con A-based glucose-responsive material was approximately 30 minutes from gel to sol and possibly longer from sol to gel. As the idea of using the glucose-responsive material to control the release of insulin secreted from the cells is that the kinetics of phase change of material are faster than those of the cells in response to stimuli, and a proper pancreatic substitute should have insulin-secretion kinetics similar to those of the normal β cells, 30 minutes of time lag is not clinically relevant. The response time could be reduced by reducing the thickness of the material barrier, and more work needs to be done in defining a proper thickness of the gel that gives a fast response time without compromising its glucose responsiveness.

Finally, con A is known to be immunogenic and toxic to cells, and it is the major glucose-sensing element in the system, thus it has to be retained permanently and maintained active so the material can undergo phase changes repeatedly. Nonetheless, with the current set up, con A tends to leak out when the material is in the sol state. Thus, to improve the biocompatibility and stability of the con A-based material, con A has to be tethered to the polysaccharide chain so it will not diffuse out. The approach

presented in Appendix 2 is a possible reaction scheme for tethering con A molecules to the polysaccharide chains, in which the PEG-con A is tethered to the polysaccharides through the covalent bonding of acrylate group on the PEG to GEMA during radical polymerization. Nonetheless, this approach as well as others requires further understanding as to whether the tethering will compromise the glucose-binding properties of con A molecules or reduce the glucose-responsiveness of the resulting hydrogels.

7.2.2.2 Improving the design of cell-material hybrid construct

The other critical component of the proposed hybrid construct is the cells being used. In this thesis, cells being used were constitutive insulin secretors, however, it might be advantageous to prevent over accumulation of insulin inside the construct by using hepatic cells that are glucose-responsive with insulin-feedback inhibition, such the one developed by Thule et al [84]. The only concern in using hepatic cells is that it is yet quite difficult to maintain primary hepatocytes ectopically. In this case, myoblasts might be a better option as the insulin source. Myoblasts are relatively easy to be retrieved as biopsy from a patient, propagated and engineered *ex vivo*, and differentiated into myotubes as a stable living protein-delivery vehicle. However, as reported in the literature [95] and in Chapter 5, myoblasts cannot differentiate into myotubes and recombinant protein secretion is decreased when these cells are encapsulated in alginate. Thereby, to facilitate the loading of stable insulin-secreting myoblasts into the hybrid construct, differentiation of myoblasts into stable myotubes in different types of biomaterials needs to be further investigated.

In terms of the design of the construct, the size of the current proposed prototype is

similar to the size of the implantable insulin pump produced by Metronics [170], which can be implanted into the peritoneal cavity. However, it appears that the transport rate of nutrients is not sufficient to maintain the cells inside the construct, thus thinner insulin-impermeable surroundings with large surface to volume ratio need to be used and evaluated. Alternatively, one could investigate the possibility of further designing the glucose-responsive material so that it can be used to coat the encapsulated cells with a thin layer, in which the pore sizes are regulated by the glucose, hence achieving glucose-regulated insulin release.

APPENDIX

A1. Improving the glucose sensitivity of con A-based material by incorporation of glucose oxidase[2]

Con A exists as tetramer at pH above 7 and as a dimer at pH below 6. Each monomer binds independently to ligand such as glucose. Thus, when con A is mixed with polysaccharides (e.g. glycogen) at neutral pH, it acts as a crosslinker and a viscous gel is resulted. When the con A-based material is exposed to a low pH environment, tetrameric con A dissociates into dimeric con A, which then causes the gel to be less crosslinked with higher insulin permeability (Figure A1 and A2).

Based on this unique property, it was hypothesized that one could improve the glucose sensitivity of the con A-based material by incorporating GluOx into the con A-based material. Similar to the design principle of glucose-responsive material based on GluOx, when the material is exposed to an increase of glucose concentration, GluOx within the hydrogel reacts with glucose, which then causes the pH within the hydrogel to drop. The lower pH within the hydrogel then leads to the dissociation of tetrameric con A into dimeric con A and causes the gel structure to be less crosslinked and more ready to be broken down by free glucose. Moreover, the binding affinity of dimeric con A is known to be smaller than that of tetrameric con A towards polysaccharides, thus the glucose concentration required to cause the gel to sol phase change is expected to be lower.

–

---

[2] Experiments were performed by the undergraduate research scholar, Angela Chu.

Preparation of con A-based material was based on the protocol described in Chapter 4 of this thesis, except various amount of GluOx (Sigma) was dissolved with con A in PBS prior to the mixing of con A with glycogen. Figure A3 shows the decrease of pH with time in wells of a 96-well plate containing con A-based materials incorporated with various amount of GluOx in different glucose concentrations. pH was measured by pH indicator strips (EMD Chemical Inc., Gibbstown, NJ). Results indicated that GluOx remained active when entrapped inside the con A-based material: it reacted with glucose and produced gluconic acid, which then lowered the pH in the surrounding environment.

As tested in a diffusion chamber experiment (experimental set up is described in Chapter 4 of this thesis) using FITC-insulin as the insulin source, incorporating GluOx into the con A-based material at 1 mg/ml did not compromise the glucose responsiveness of the con A-based material. Con A-based material with or without GluOx exhibited similar changes of FITC-insulin diffuse rate when materials were exposed to a 4% glucose step up at t=2 hour and a glucose step down at t=5 hour (Figure A4). However, in experiments having a lower glucose step change that is closer to physiological range (e.g. 0.5 % at t=2 hour, Figure A5), no significant changes in FITC-insulin flux was observed for con A-based material both with or without GluOx.

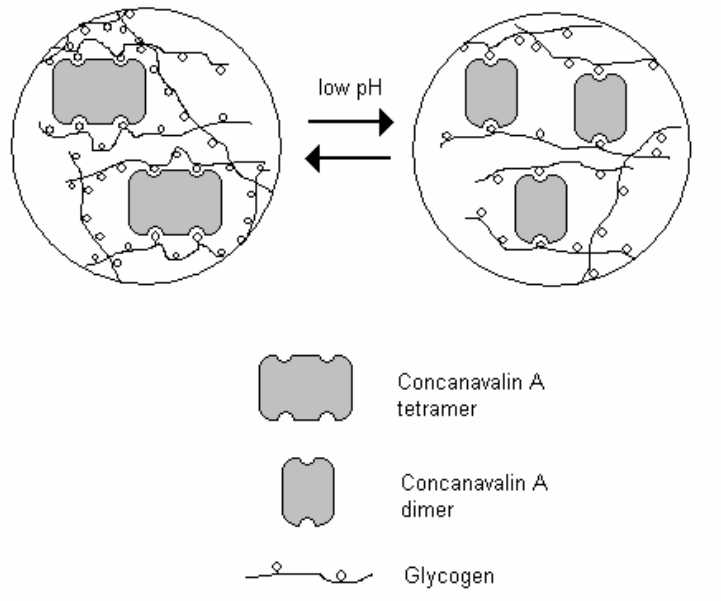


Figure A 1. Schematic of con A-based material exposed to low pH environment. Con A dissociates from tetrameric into dimeric structure, which causes the gel to be less crosslinked.

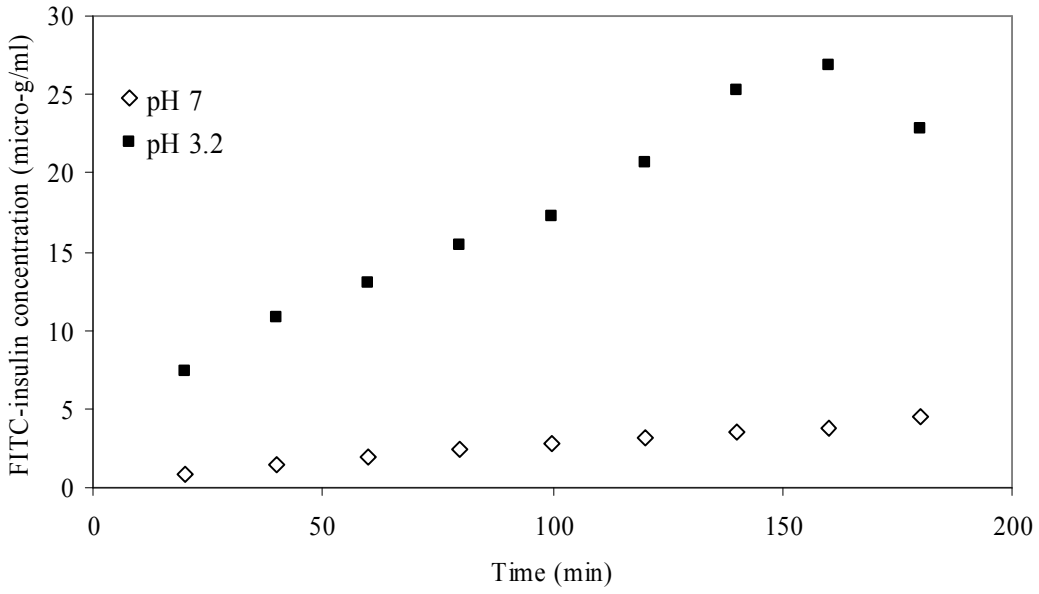
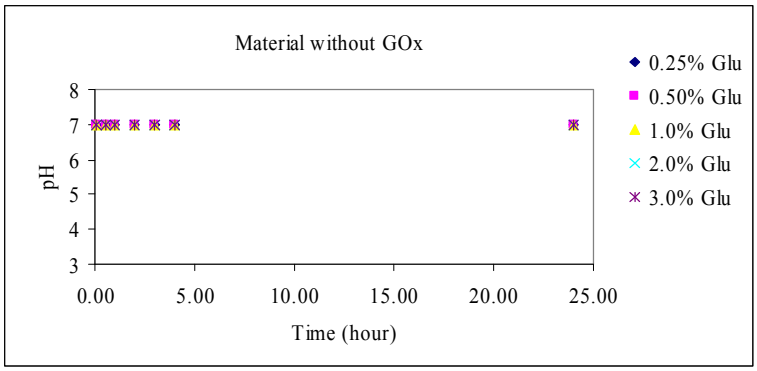
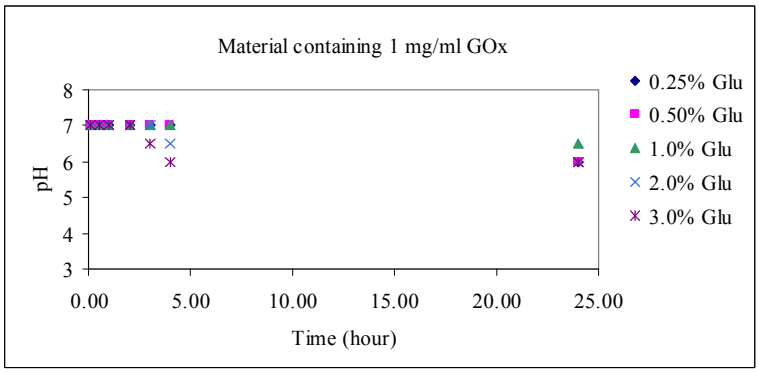


Figure A 2. Accumulation of FITC-insulin in the receiver compartments of two diffusion apparatus (experimental set up is described in Chapter 4) containing FITC-insulin in the donor compartments at different pH. At pH 3.2, due to the formation of dimeric con A instead of tetrameric con A, con A-based material was less crosslinked with higher FITC-insulin permeability.

(a)



(b)



(c)

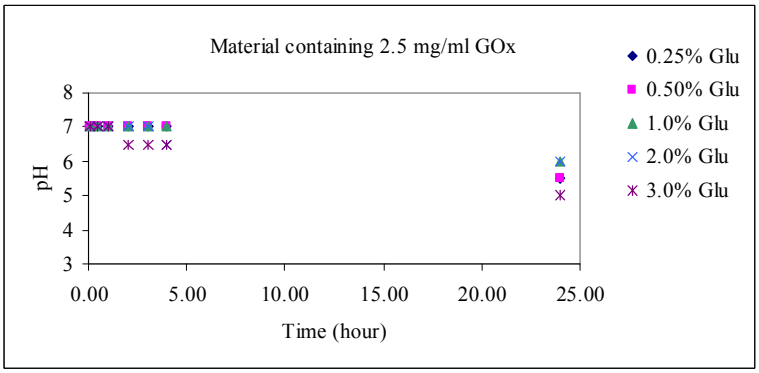
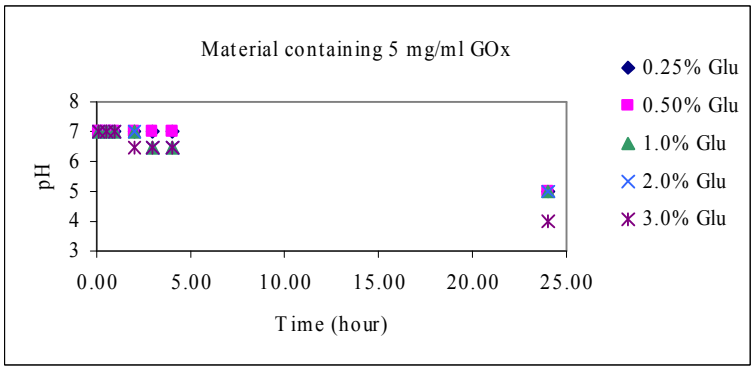


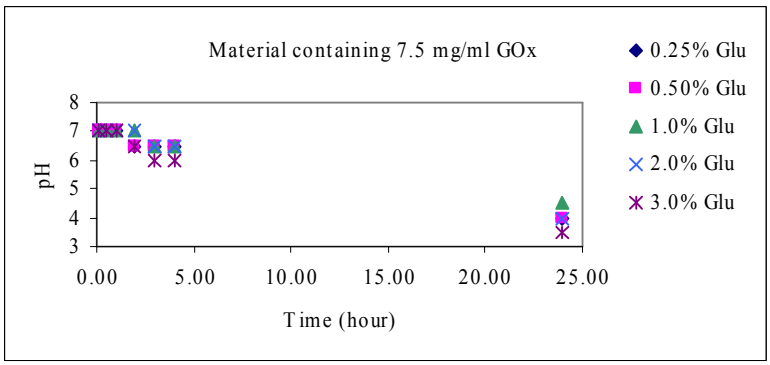
Figure A 3. Changes of pH with time in different glucose solution containing con A-based material incorporated with (a) 0 mg/ml (b) 1 mg/ml (c) 2.5 mg/ml (d) 5 mg/ml (e) 7.5 mg/ml (f) 10 mg/ml of GluOx.

Figure A 3. (continued).

(d)



(e)



(f)

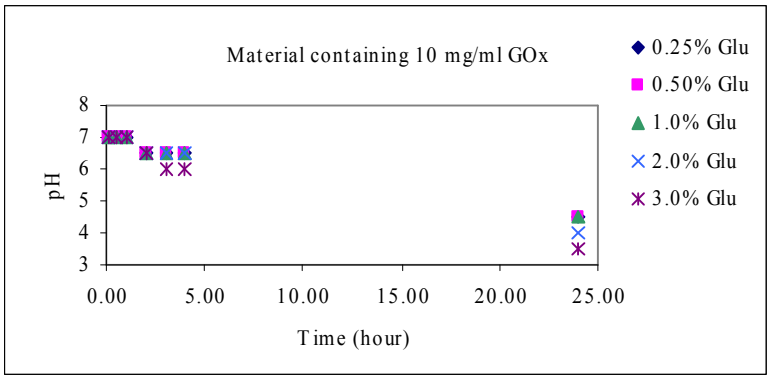


Figure A 3. Changes of pH with time in different glucose solution containing con A-based material incorporated with (a) 0 mg/ml (b) 1 mg/ml (c) 2.5 mg/ml (d) 5 mg/ml (e) 7.5 mg/ml (f) 10 mg/ml of GluOx.

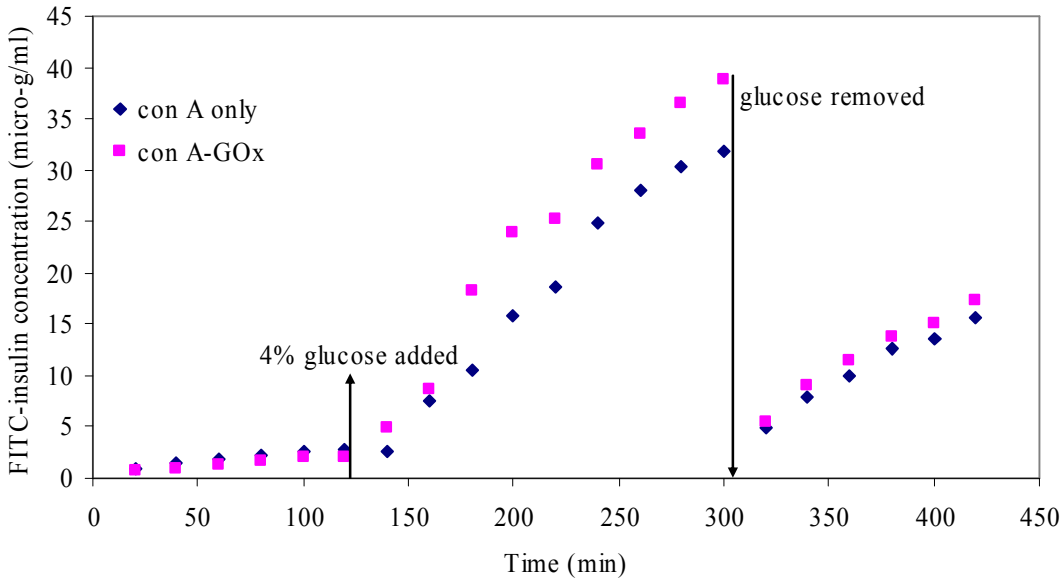


Figure A 4. Accumulation of FITC-insulin in the receiver compartments of two diffusion apparatus containing FITC-insulin in the donor compartment. Con A-based material with or without 1 mg/ml of GluOx were in gel state at t=0 hour.

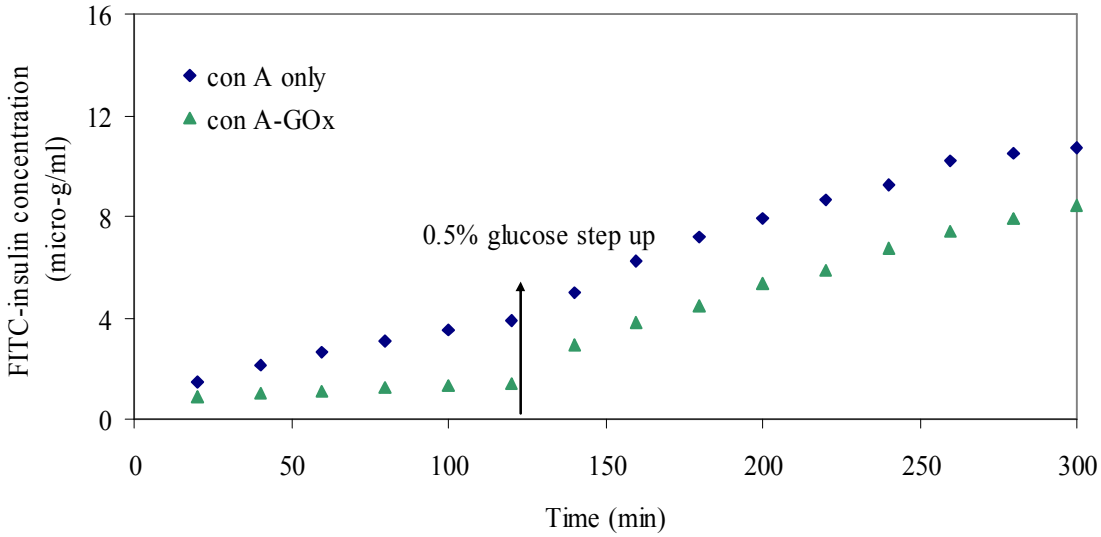


Figure A 5. Accumulation of FITC-insulin in the receiver compartments of two diffusion apparatus containing FITC-insulin in the donor compartment. Con A-based material with or without 1 mg/ml of GluOx were in gel state at t=0.

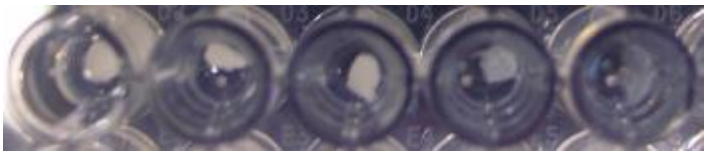
A2. Glucose responsive material based on con A and GEMA

Preparation of poly(GEMA) and con A-based glucose-responsive material was based on the general protocol published by Miyata et al [142]. Briefly, 0.1 ml of glucosyloxyethyl methacrylate (GEMA) (Nippon Fine Chemcial, Osaka, Japan) mixed with 1.9 ml of PBS was used to dissolve 15 mg of con A, and 0.01 g of initiator, 2,2'-Axobis(2-amidinopropance) dihydrochloride (AIBA) (Sigma). To remove radical scavenger such as oxygen, the mixture was purged with nitrogen for 1 hour prior to polymerization. After exposing the mixture to UV light for 30 minutes, a whitish gel was resulted. The gel appeared to be more elastic than the material formed with glycogen and con A. To test whether the con A-poly(GEMA) gel exhibit glucose responsiveness, the gel was cut into pieces of similar size and loaded into a well of a 24-well plate. 1 ml of glucose solution (220, 110, 55, 25 and 0 mM) was then added into each well. Pictures were taken prior to and an hour after glucose solution was added (Figure A6 a and b). Results indicated that the material turned into a sol at glucose concentrations between 110 and 220 mM.

To prevent con A leakage during phase changes, a reaction scheme was proposed to tether con A molecules to polysaccharides. In this, Acryl-PEG-con A, prepared as described in chapter 6, was used to mix with GEMA and AIBA in PBS in the same composition as described above. The idea was that during polymerization, the acrylate group on a PEGylated con A would react with the acrylate group on a polyGEMA, and thus tethering con A to the polysaccharides. However, no gel formation was observed in reactions using PEG 2.5-con A or PEG 5-con A. More experiments are needed to understand what led to the loss of gel formation.

(a) T=0 hour

(b) T=1 hour



0 0.5% 1% 2% 4% Glucose

Figure A 6. Con A-GEMA glucose responsive material before (a) and one hour after exposing to glucose solution (b).

REFERENCES

1. http://www.diabetes.org/diabetes-statistics/national-diabetes-fact-sheet.jsp

2. The Diabetes Control and Complications Trial Research Group: The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus, N. Engl. J. Med. 329(1993) 977-986.

3. A.M. Shapiro, J.R. Lakey, E.A. Ryan, G.S. Korbutt, E. Toth, G.L. Warnock, N.M. Kneteman, R.V. Rajotte, Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen, N Engl J Med. 343(4) (2000) 230-238.

4. J. Shapiro, Eighty years after insulin: parallels with modern islet transplantation, Cmaj. 167(12) (2002) 1398-1400.

5. S. Efrat, S. Linde, H. Kofod, D. Spector, M. Delannoy, S. Grant, D. Hanahan, S. Baekkeskov, Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene, Proc Natl Acad Sci U S A. 85(23) (1988) 9037-9041.

6. R. Chen, M. Meseck, R.C. McEvoy, S.L. Woo, Glucose-stimulated and self-limiting insulin production by glucose 6-phosphatase promoter driven insulin expression in hepatoma cells, Gene Ther. 7(21) (2000) 1802-1809.

7. L. Gros, E. Riu, L. Montoliu, M. Ontiveros, L. Lebrigand, F. Bosch, Insulin production by engineered muscle cells, Hum Gene Ther. 10(7) (1999) 1207-1217.

8. J.J. Kim, K. Park, Glucose-binding property of pegylated concanavalin A, Pharm Res. 18(6) (2001) 794-799.

9. American Diabetes Association: Economic costs of diabetes in the U.S. in 2002, Diabetes Care. 26(2003) 917-932.

10. Adverse events and their association with treatment regimens in the diabetes control and complications trial, Diabetes Care. 18(11) (1995) 1415-1427.

11. G. Emilien, J.M. Maloteaux, M. Ponchon, Pharmacological management of diabetes: recent progress and future perspective in daily drug treatment, Pharmacol Ther. 81(1) (1999) 37-51.

12. E. Stephens, M. Riddle, Evolving approaches to intensive insulin therapy in type 1 diabetes: multiple daily injections, insulin pumps and new methods of monitoring, Rev Endocr Metab Disord. 4(4) (2003) 325-334.

13. J. Jaremko, O. Rorstad, Advances toward the implantable artificial pancreas for treatment of diabetes, Diabetes Care. 21(3) (1998) 444-450.

14. P. Brunetti, M. Orsini Federici, M. Massi Benedetti, The artificial pancreas, Artif Cells Blood Substit Immobil Biotechnol. 31(2) (2003) 127-138.

15. L. Brown, E. Mathiowitz, D. Brandenburg, R. Langer, Enzymatically controlled drug delivery, Proc. Natl. Acad. Sci. 85(1988) 2403-2406.

16. L.R. Brown, E.R. Edelman, F. Fischel-Ghodsian, R. Langer, Characterization of glucose-mediated insulin release from implantable polymers, Journal of Pharmaceutical Sciences. 85(12) (1996) 1341-1345.

17. C. Hassan, F. Doyle III, N. Peppas, Dynamic behavior of glucose-responsive poly(methacrylic acid-g-ethylene glycol) hydrogels, Macromolecules. 30(1997) 6166-6173.

18. Y. Ito, M. Casolaro, K. Kono, Y. Imanishi, An insulin-releasing system that is responsive to glucose, Journal of Controlled Release. 10(1989) 195-203.

19. L.Y. Chu, Y. Li, J.H. Zhu, H.D. Wang, Y.J. Liang, Control of pore size and permeability of a glucose-responsive gating membrane for insulin delivery, J Control Release. 97(1) (2004) 43-53.

20. J. Kost, T.A. Horbett, B.D. Ratner, M. Singh, Glucose-sensitive membranes containing glucose oxidase: activity, swelling, and permeability studies, J Biomed Mater Res. 19(9) (1985) 1117-1133.

21. T. Traitel, Y. Cohen, J. Kost, Characterization of glucose-sensitive insulin release systems in simulated in vivo conditions, Biomaterials. 21(16) (2000) 1679-1687.

22. R.A. Siegel, *pH-sensitive gels: swelling equilibria, kinetics and applications for drug delivery*, in *Pulsed and self-regulated drug delivery*, J. Kost, Editor. 1990, CRC press: Boca Raton, FL. p. 129-157.

23. J. Heller, *Use of enzymes and bioerodible polymers in self-regulated and triggered drug delivery systems*, in *Pulsed and self-regulated drug delivery*, J. Kost, Editor. 1990, CRC Press: Boca Raton, FL. p. 93-108.

24. T. Uchiyama, Y. Kiritoshi, J. Watanabe, K. Ishihara, Degradation of phospholipid polymer hydrogel by hydrogen peroxide aiming at insulin release device, Biomaterials. 24(28) (2003) 5183-5190.

25. D.-Y. Jung, J.J. Magda, I.S. Han, Catalase Effects on Glucose-Sensitive Hydrogels, Macromolecules. 33(2000) 3332-3336.

26. D. Shiino, Y. murata, D. Kataoka, Y. Koyama, M. Yokoyama, T. Okano, Y. Sakurai, Preparation and characterization of a glucose-responsive insulin-releasing polymer device, Biomaterial. 15(2) (1994) 121-128.

27. S. Kitano, Y. Koyama, k. Kataoka, T. Okano, Y. Sakurai, A novel drug delivery systme utilizing a glucose responsive polymer complex between poly(vinyl alcohol) and poly(N-vinyl-2-pyrrolidone) with a phenyl boronic acid moiety, Journal of Controlled Release. 19(1992) 162-170.

28. K. Kataoka, H. Miyazaki, M. Bunya, T. Okano, Y. Sakurai, Totally synthetic polymer gels responding to external glucose concentration: Their preparation and application to on-off regulation of insulin release, J. Am.Chem. Soc. 120(1998) 12694-12695.

29. K. Kataoka, H. Miyazaki, K. Miyaji, I. Hisamitsu, K. Tanaka, T. Okano, Y. Sakurai, Novel glucose-sensitive polymers and hydrogels based on phenylborate containing polymers, Polymer Preprints. 35(2) (1994) 393-394.

30. I. Hisamitsu, K. Kataoka, T. Okano, Y. Sakurai, GLucose-responsive gle from phenylborate polymer and poly(vinyl alcohol): Prompt response at physiological pH through the interaction of borate with amino group in the gel, Pharm Res. 14(3) (1997) 289-293.

31. A. Matsumoto, R. Yoshida, K. Kataoka, Glucose-responsive polymer gel bearing phenylborate derivative as a glucose-sensing moiety operating at the physiological pH, Biomacromolecules. 5(3) (2004) 1038-1045.

32. H. Bittiger, H.P. Schnebli, *Concanavalin A as a tool*. 1976: John Wiley & Sons.

33. C.M. Pai, Y.H. Bae, E.J. Mack, D.E. Wilson, S.W. Kim, Concanavalin A microspheres for a self-regulating insulin delivery system, J. Pharmaceutical Sciences. 81(6) (1992) 532-536.

34. A.A. Obaidat, K. Park, Characterization of protein release through glucose-sensitive hydrogel membranes, Biomaterials. 18(11) (1997) 801-806.

35. S. Tanna, M.J. Taylor, Characterization of model solute and insulin delivery across covalently modified lectin-polysaccharide gels sensitive to glucose, Pharm. Pharmacol. Commun. 4(1998) 117-122.

36. K. Nakamae, T. Miyata, A. Jikihara, A.S. Hoffman, Formation of poly(glucosyloxyethyl methacrylate)-concanavalin A complex and its glucose-sensitivity, J Biomater Sci Polym Ed. 6(1) (1994) 79-90.

37. T. Miyagi, T. Takehara, T. Tatsumi, T. Suzuki, M. Jinushi, Y. Kanazawa, N. Hiramatsu, T. Kanto, S. Tsuji, M. Hori, N. Hayashi, Concanavalin a injection activates intrahepatic innate immune cells to provoke an antitumor effect in

murine liver, Hepatology. 40(5) (2004) 1190-1196.

38. S. Tanna, T. Sahota, J. Clark, M.J. Taylor, A covalently stabilised glucose responsive gel formulation with a Carbopol carrier, J Drug Target. 10(5) (2002) 411-418.

39. S. Tanna, T. Sahota, J. Clark, M.J. Taylor, Covalent coupling of concanavalin A to a Carbopol 934P and 941P carrier in glucose-sensitive gels for delivery of insulin, J Pharm Pharmacol. 54(11) (2002) 1461-1469.

40. M. Tang, R. Zhang, A. Bowyer, R. Eisenthal, J. Hubble, A reversible hydrogel membrane for controlling the delivery of macromolecules, Biotechnol Bioeng. 82(1) (2003) 47-53.

41. K. Ohneda, H. Ee, M. German, Regulation of insulin gene transcription, Semin Cell Dev Biol. 11(4) (2000) 227-233.

42. M.S. German, Glucose sensing in pancreatic islet beta cells: the key role of glucokinase and the glycolytic intermediates, Proc Natl Acad Sci U S A. 90(5) (1993) 1781-1785.

43. B. Wicksteed, C. Alarcon, I. Briaud, M.K. Lingohr, C.J. Rhodes, Glucose-induced translational control of proinsulin biosynthesis is proportional to preproinsulin mRNA levels in islet beta-cells but not regulated via a positive feedback of secreted insulin, J Biol Chem. 278(43) (2003) 42080-42090.

44. M. Welsh, D.A. Nielsen, A.J. MacKrell, D.F. Steiner, Control of insulin gene expression in pancreatic beta-cells and in an insulin-producing cell line, RIN-5F cells. II. Regulation of insulin mRNA stability, J Biol Chem. 260(25) (1985) 13590-13594.

45. M. Welsh, N. Scherberg, R. Gilmore, D.F. Steiner, Translational control of insulin biosynthesis. Evidence for regulation of elongation, initiation and signal-recognition-particle-mediated translational arrest by glucose, Biochem J. 235(2) (1986) 459-467.

46. M. Gilligan, G.I. Welsh, A. Flynn, I. Bujalska, T.A. Diggle, R.M. Denton, C.G. Proud, K. Docherty, Glucose stimulates the activity of the guanine nucleotide-exchange factor eIF-2B in isolated rat islets of Langerhans, J Biol Chem. 271(4) (1996) 2121-2125.

47. C. Alarcon, B. Lincoln, C.J. Rhodes, The biosynthesis of the subtilisin-related proprotein convertase PC3, but no that of the PC2 convertase, is regulated by glucose in parallel to proinsulin biosynthesis in rat pancreatic islets, J Biol Chem. 268(6) (1993) 4276-4280.

48. D. Kaelin, A.E. Renold, G.W. Sharp, Glucose stimulated proinsulin biosynthesis rates of turn off after cessation of the stimulus, Diabetologia. 14(5) (1978) 329-335.

49. F.C. Schuit, P.A. In't Veld, D.G. Pipeleers, Glucose stimulates proinsulin biosynthesis by a dose-dependent recruitment of pancreatic beta cells, Proc Natl Acad Sci U S A. 85(11) (1988) 3865-3869.

50. M.D. Meglasson, F.M. Matschinsky, Pancreatic islet glucose metabolism and regulation of insulin secretion, Diabetes Metab Rev. 2(3-4) (1986) 163-214.

51. J.T. Deeney, M. Prentki, B.E. Corkey, Metabolic control of beta-cell function, Semin Cell Dev Biol. 11(4) (2000) 267-275.

52. T.K. Bratanova-Tochkova, H. Cheng, S. Daniel, S. Gunawardana, Y.J. Liu, J. Mulvaney-Musa, T. Schermerhorn, S.G. Straub, H. Yajima, G.W. Sharp, Triggering and augmentation mechanisms, granule pools, and biphasic insulin secretion, Diabetes. 51 Suppl 1(2002) S83-90.

53. S.G. Straub, G.W.G. Sharp, Glucose-stimulated signaling pathways in biphasic insulin secretion, Diabetes/Metabolism Research and Reviews. 18(2002) 451-463.

54. E.A. Ryan, J.R. Lakey, R.V. Rajotte, G.S. Korbutt, T. Kin, S. Imes, A. Rabinovitch, J.F. Elliott, D. Bigam, N.M. Kneteman, G.L. Warnock, I. Larsen, A.M. Shapiro, Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol, Diabetes. 50(4) (2001) 710-719.

55. J.R. Lakey, P.W. Burridge, A.M. Shapiro, Technical aspects of islet preparation and transplantation, Transpl Int. 16(9) (2003) 613-632.

56. L.J. van der Laan, C. Lockey, B.C. Griffeth, F.S. Frasier, C.A. Wilson, D.E. Onions, B.J. Hering, Z. Long, E. Otto, B.E. Torbett, D.R. Salomon, Infection by porcine endogenous retrovirus after islet xenotransplantation in SCID mice, Nature. 407(6800) (2000) 90-94.

57. D.W. Gray, An overview of the immune system with specific reference to membrane encapsulation and islet transplantation, Ann N Y Acad Sci. 944(2001) 226-239.

58. V.K. Ramiya, M. Maraist, K.E. Arfors, D.A. Schatz, A.B. Peck, J.G. Cornelius, Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells, Nat Med. 6(3) (2000) 278-282.

59. S. Bonner-Weir, M. Taneja, G.C. Weir, K. Tatarkiewicz, K.H. Song, A. Sharma, J.J. O'Neil, In vitro cultivation of human islets from expanded ductal tissue, Proc Natl Acad Sci U S A. 97(14) (2000) 7999-8004.

60. D.H. Sachs, S. Bonner-Weir, New islets from old, Nat Med. 6(3) (2000) 250-251.

61. S. Assady, G. Maor, M. Amit, J. Itskovitz-Eldor, K.L. Skorecki, M. Tzukerman, Insulin production by human embryonic stem cells, Diabetes. 50(8) (2001) 1691-1697.

62. N. Lumelsky, O. Blondel, P. Laeng, I. Velasco, R. Ravin, R. McKay, Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets, Science. 292(5520) (2001) 1389-1394.

63. Y. Hori, I.C. Rulifson, B.C. Tsai, J.J. Heit, J.D. Cahoy, S.K. Kim, Growth inhibitors promote differentiation of insulin-producing tissue from embryonic stem cells, Proc Natl Acad Sci U S A. 99(25) (2002) 16105-16110.

64. B. Soria, E. Roche, G. Berna, T. Leon-Quinto, J.A. Reig, F. Martin, Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice, Diabetes. 49(2) (2000) 157-162.

65. J.A. Thomson, J. Itskovitz-Eldor, S.S. Shapiro, M.A. Waknitz, J.J. Swiergiel, V.S. Marshall, J.M. Jones, Embryonic stem cell lines derived from human blastocysts, Science. 282(5391) (1998) 1145-1147.

66. B.E. Reubinoff, M.F. Pera, C.Y. Fong, A. Trounson, A. Bongso, Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro, Nat Biotechnol. 18(4) (2000) 399-404.

67. S. Efrat, S. Linde, H. Kofod, D. Spector, M. Delannoy, S. Grant, D. Hanahan, S. Baekkeskov, Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene, Proc Natl Acad Sci U S A. 85(23) (1988) 9037-9041.

68. S. Efrat, M. Surana, N. Fleischer, Glucose induces insulin gene transcription in a murine pancreatic beta- cell line, J Biol Chem. 266(17) (1991) 11141-11143.

69. D. Knaack, D.M. Fiore, M. Surana, M. Leiser, M. Laurance, D. Fusco-DeMane, O.D. Hegre, N. Fleischer, S. Efrat, Clonal insulinoma cell line that stably maintains correct glucose responsiveness, Diabetes. 43(12) (1994) 1413-1417.

70. S. Efrat, D. Fusco-DeMane, H. Lemberg, O. al Emran, X. Wang, Conditional transformation of a pancreatic beta-cell line derived from transgenic mice expressing a tetracycline-regulated oncogene, Proc Natl Acad Sci U S A. 92(8) (1995) 3576-3580.

71. C. Stabler, K. Wilks, A. Sambanis, I. Constantinidis, The effects of alginate composition on encapsulated betaTC3 cells, Biomaterials. 22(11) (2001) 1301-1310.

72. D.J. Groskreutz, M.X. Sliwkowski, C.M. Gorman, Genetically engineered proinsulin constitutively processed and secreted as mature, active insulin, J Biol Chem. 269(8) (1994) 6241-6245.

73. M. Yanagita, H. Hoshino, K. Nakayama, T. Takeuchi, Processing of mutated proinsulin with tetrabasic cleavage sites to mature insulin reflects the expression of furin in nonendocrine cell lines, Endocrinology. 133(2) (1993) 639-644.

74. M. Yanagita, K. Nakayama, T. Takeuchi, Processing of mutated proinsulin with tetrabasic cleavage sites to bioactive insulin in the non-endocrine cell line, COS-7, FEBS Lett. 311(1) (1992) 55-59.

75. H.C. Lee, S.J. Kim, K.S. Kim, H.C. Shin, J.W. Yoon, Remission in models of type 1 diabetes by gene therapy using a single-chain insulin analogue, Nature. 408(6811) (2000) 483-488.

76. J. Wahren, K. Ekberg, J. Johansson, M. Henriksson, A. Pramanik, B.L. Johansson, R. Rigler, H. Jornvall, Role of C-peptide in human physiology, Am J Physiol Endocrinol Metab. 278(5) (2000) E759-768.

77. R. Burcelin, W. Dolci, B. Thorens, Glucose sensing by the hepatoportal sensor is GLUT2-dependent: in vivo analysis in GLUT2-null mice, Diabetes. 49(10) (2000) 1643-1648.

78. M.A. Magnuson, Tissue-specific regulation of glucokinase gene expression, J Cell Biochem. 48(2) (1992) 115-121.

79. D. Lu, H. Tamemoto, H. Shibata, I. Saito, T. Takeuchi, Regulatable production of insulin from primary-cultured hepatocytes: insulin production is up-regulated by glucagon and cAMP and down-regulated by insulin, Gene Ther. 5(7) (1998) 888-895.

80. F. Cournarie, D. Azzout-Marniche, M. Foretz, C. Guichard, P. Ferre, F. Foufelle, The inhibitory effect of glucose on phosphoenolpyruvate carboxykinase gene expression in cultured hepatocytes is transcriptional and requires glucose metabolism, FEBS Lett. 460(3) (1999) 527-532.

81. D.K. Scott, R.M. O'Doherty, J.M. Stafford, C.B. Newgard, D.K. Granner, The repression of hormone-activated PEPCK gene expression by glucose is insulin-independent but requires glucose metabolism, J Biol Chem. 273(37) (1998) 24145-24151.

82. R. Chen, M.L. Meseck, S.L. Woo, Auto-regulated hepatic insulin gene expression in type 1 diabetic rats, Mol Ther. 3(4) (2001) 584-590.

83. P.M. Thule, J.M. Liu, Regulated hepatic insulin gene therapy of STZ-diabetic rats, Gene Ther. 7(20) (2000) 1744-1752.

84. P.M. Thule, J. Liu, L.S. Phillips, Glucose regulated production of human insulin in rat hepatocytes, Gene Ther. 7(3) (2000) 205-214.

85. D.E. Olson, S.A. Paveglio, P.U. Huey, M.H. Porter, P.M. Thule, Glucose-responsive hepatic insulin gene therapy of spontaneously diabetic BB/Wor rats, Hum Gene Ther. 14(15) (2003) 1401-1413.

86. S.C. Tang, A. Sambanis, Preproinsulin mRNA engineering and its application to the regulation of insulin secretion from human hepatomas, FEBS Lett. 537(1-3) (2003) 193-197.

87. P. Gregorevic, M.J. Blankinship, J.M. Allen, R.W. Crawford, L. Meuse, D.G. Miller, D.W. Russell, J.S. Chamberlain, Systemic delivery of genes to striated muscles using adeno-associated viral vectors, Nat Med. 10(8) (2004) 828-834.

88. N. Deglon, B. Heyd, S.A. Tan, J.M. Joseph, A.D. Zurn, P. Aebischer, Central nervous system delivery of recombinant ciliary neurotrophic factor by polymer encapsulated differentiated C2C12 myoblasts, Hum Gene Ther. 7(17) (1996) 2135-2146.

89. E. Regulier, B.L. Schneider, N. Deglon, Y. Beuzard, P. Aebischer, Continuous delivery of human and mouse erythropoietin in mice by genetically engineered polymer encapsulated myoblasts, Gene Ther. 5(8) (1998) 1014-1022.

90. M. Roman, J.H. Axelrod, Y. Dai, R.K. Naviaux, T. Friedmann, I.M. Verma, Circulating human or canine factor IX from retrovirally transduced primary myoblasts and established myoblast cell lines grafted into murine skeletal muscle, Somat Cell Mol Genet. 18(3) (1992) 247-258.

91. Y. Dai, M. Roman, R.K. Naviaux, I.M. Verma, Gene therapy via primary myoblasts: long-term expression of factor IX protein following transplantation in vivo, Proc Natl Acad Sci U S A. 89(22) (1992) 10892-10895.

92. C. Garcia-Martin, M.K. Chuah, A. Van Damme, K.E. Robinson, B. Vanzieleghem, J.M. Saint-Remy, D. Gallardo, F.A. Ofosu, T. Vandendriessche, G. Hortelano, Therapeutic levels of human factor VIII in mice implanted with encapsulated cells: potential for gene therapy of haemophilia A, J Gene Med. 4(2) (2002) 215-223.

93. H. Vandenburgh, M. Del Tatto, J. Shansky, L. Goldstein, K. Russell, N. Genes, J. Chromiak, S. Yamada, Attenuation of skeletal muscle wasting with recombinant human growth hormone secreted from a tissue-engineered bioartificial muscle, Hum Gene Ther. 9(17) (1998) 2555-2564.

94. Y. Lu, J. Shansky, M. Del Tatto, P. Ferland, S. McGuire, J. Marszalkowski, M. Maish, R. Hopkins, X. Wang, P. Kosnik, M. Nackman, A. Lee, B. Creswick, H. Vandenburgh, Therapeutic potential of implanted tissue-engineered bioartificial muscles delivering recombinant proteins to the sheep heart, Ann N Y Acad Sci. 961(2002) 78-82.

95. A.A. Li, N.C. MacDonald, P.L. Chang, Effect of growth factors and extracellular matrix materials on the proliferation and differentiation of microencapsulated myoblasts, J Biomater Sci Polym Ed. 14(6) (2003) 533-549.

96. K. Yamasaki, T. Sasaki, M. Nemoto, Y. Eto, N. Tajima, Differentiation-induced insulin secretion from nonendocrine cells with engineered human proinsulin cDNA, Biochem Biophys Res Commun. 265(2) (1999) 361-365.

97. V.M. Rivera, X. Wang, S. Wardwell, N.L. Courage, A. Volchuk, T. Keenan, D.A. Holt, M. Gilman, L. Orci, F. Cerasoli, Jr., J.E. Rothman, T. Clackson, Regulation of protein secretion through controlled aggregation in the endoplasmic reticulum, Science. 287(5454) (2000) 826-830.

98. S.D. Hughes, C. Quaade, J.L. Milburn, L. Cassidy, C.B. Newgard, Expression of normal and novel glucokinase mRNAs in anterior pituitary and islet cells, J Biol Chem. 266(7) (1991) 4521-4530.

99. S.D. Hughes, J.H. Johnson, C. Quaade, C.B. Newgard, Engineering of glucose-stimulated insulin secretion and biosynthesis in non-islet cells, Proc Natl Acad Sci U S A. 89(2) (1992) 688-692.

100. R.N. Faradji, E. Havari, Q. Chen, J. Gray, K. Tornheim, B.E. Corkey, R.C. Mulligan, M.A. Lipes, Glucose-induced toxicity in insulin-producing pituitary cells that coexpress GLUT2 and glucokinase. Implications for metabolic engineering, J Biol Chem. 276(39) (2001) 36695-36702.

101. J. Schirra, M. Katschinski, C. Weidmann, T. Schafer, U. Wank, R. Arnold, B. Goke, Gastric emptying and release of incretin hormones after glucose ingestion in humans, J Clin Invest. 97(1) (1996) 92-103.

102. A.T. Cheung, B. Dayanandan, J.T. Lewis, G.S. Korbutt, R.V. Rajotte, M. Bryer-Ash, M.O. Boylan, M.M. Wolfe, T.J. Kieffer, Glucose-dependent insulin release from genetically engineered K cells, Science. 290(5498) (2000) 1959-1962.

103. S.C. Tang, A. Sambanis, Development of genetically engineered human intestinal cells for regulated insulin secretion using rAAV-mediated gene transfer, Biochem Biophys Res Commun. 303(2) (2003) 645-652.

104. T. Miyata, T. Uragami, K. Nakamae, Biomolecule-sensitive hydrogels, Adv Drug

Deliv Rev. 54(1) (2002) 79-98.

105. J. Kost, R. Langer, Responsive polymeric delivery systems, Adv Drug Deliv Rev. 46(1-3) (2001) 125-148.

106. Y. Qiu, K. Park, Environment-sensitive hydrogels for drug delivery, Adv Drug Deliv Rev. 53(3) (2001) 321-339.

107. A. Shapiro, J. Lakey, E. Ryan, G. Korbutt, E. Toth, G. Warnock, N. Kneteman, R. Raiotte, Islet transplantation in seven patinets with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen, N Engl J Med. 343(2000) 230-238.

108. E.A. Ryan, B.W. Paty, P.A. Senior, A.M. Shapiro, Risks and side effects of islet transplantation, Curr Diab Rep. 4(4) (2004) 304-309.

109. B. Hirshberg, K.I. Rother, B.J. Digon, 3rd, J. Lee, J.L. Gaglia, K. Hines, E.J. Read, R. Chang, B.J. Wood, D.M. Harlan, Benefits and risks of solitary islet transplantation for type 1 diabetes using steroid-sparing immunosuppression: the National Institutes of Health experience, Diabetes Care. 26(12) (2003) 3288-3295.

110. T. Loudovaris, T.E. Mandel, B. Charlton, CD4+ T cell mediated destruction of xenografts within cell-impermeable membranes in the absence of CD8+ T cells and B cells, Transplantation. 61(12) (1996) 1678-1684.

111. J. Brauker, L.A. Martinson, S.K. Young, R.C. Johnson, Local inflammatory response around diffusion chambers containing xenografts. Nonspecific destruction of tissues and decreased local vascularization, Transplantation. 61(12) (1996) 1671-1677.

112. N. Fleischer, C. Chen, M. Surana, M. Leiser, L. Rossetti, W. Pralong, S. Efrat, Functional analysis of a conditionally transformed pancreatic beta-cell line, Diabetes. 47(9) (1998) 1419-1425.

113. I. Constantinidis, I. Rask, R.C. Long, Jr., A. Sambanis, Effects of alginate composition on the metabolic, secretory, and growth characteristics of entrapped beta TC3 mouse insulinoma cells, Biomaterials. 20(21) (1999) 2019-2027.

114. C. Stabler, K. Wilks, A. Sambanis, I. Constantinidis, The effects of alginate composition on encapsulated betaTC3 cells, Biomaterials. 22(11) (2001) 1301-1310.

115. C.L. Stabler, A. Sambanis, I. Constantinidis, Effects of alginate composition on the growth and overall metabolic activity of betaTC3 cells, Ann N Y Acad Sci. 961(2002) 130-133.

116. K.K. Papas, R.C. Long, Jr., A. Sambanis, I. Constantinidis, Development of a bioartificial pancreas: II. Effects of oxygen on long-term entrapped betaTC3 cell cultures, Biotechnol Bioeng. 66(4) (1999) 231-237.
117. R.A. Ritzel, J.D. Veldhuis, P.C. Butler, Glucose stimulates pulsatile insulin secretion from human pancreatic islets by increasing secretory burst mass: dose-response relationships, J Clin Endocrinol Metab. 88(2) (2003) 742-747.

118. J.C. Henquin, N. Ishiyama, M. Nenquin, M.A. Ravier, J.C. Jonas, Signals and pools underlying biphasic insulin secretion, Diabetes. 51 Suppl 1(2002) S60-67.

119. Y.H. Ma, J. Wang, G.G. Rodd, J.L. Bolaffi, G.M. Grodsky, Differences in insulin secretion between the rat and mouse: role of cAMP, Eur J Endocrinol. 132(3) (1995) 370-376.

120. W.S. Zawalich, K.C. Zawalich, Regulation of insulin secretion by phospholipase C, Am J Physiol. 271(3 Pt 1) (1996) E409-416.

121. I.R. Sweet, D.L. Cook, R.W. Wiseman, C.J. Greenbaum, A. Lernmark, S. Matsumoto, J.C. Teague, K.A. Krohn, Dynamic perifusion to maintain and assess isolated pancreatic islets, Diabetes Technol Ther. 4(1) (2002) 67-76.

122. A. Fritsche, N. Stefan, E. Hardt, S. Schutzenauer, H. Haring, M. Stumvoll, A novel hyperglycaemic clamp for characterization of islet function in humans: assessment of three different secretagogues, maximal insulin response and reproducibility, Eur J Clin Invest. 30(5) (2000) 411-418.

123. R.A. DeFronzo, J.D. Tobin, R. Andres, Glucose clamp technique: a method for quantifying insulin secretion and resistance, Am J Physiol. 237(3) (1979) E214-223.

124. R. Coger, M. Toner, P. Moghe, R.M. Ezzell, M.L. Yarmush, Hepatocyte aggregation and reorganization of EHS matrix gel, Tissue Engineering. 3(4) (1997) 375-390.

125. H.A. Leddy, H.A. Awad, F. Guilak, Molecular diffusion in tissue-engineered cartilage constructs: Effects of scaffold material, time, and culture conditions, J Biomed Mater Res. 70B(2) (2004) 397-406.

126. E. Tziampazis, A. Sambanis, Tissue engineering of a bioartificial pancreas: modeling the cell environment and device function, Biotechnol Prog. 11(2) (1995) 115-126.

127. Y. Zimmer, D. Milo-Landesman, A. Svetlanov, S. Efrat, Genes induced by growth arrest in a pancreatic beta cell line: identification by analysis of cDNA arrays, FEBS Lett. 457(1) (1999) 65-70.

128. N.E. Simpson, N. Khokhlova, J.A. Oca-Cossio, S.S. Mcfarlane, C.P. Simpson, I. Constantinidis, Effects of growth regulation on conditionally-transformed alginate-entrapped insulin secreting cell lines in vitro.
129. M. Yanagita, K. Nakayama, T. Takeuchi, Processing of mutated proinsulin with tetrabasic cleavage sites to bioactive insulin in the non-endocrine cell line, COS-7, FEBS Lett. 311(1) (1992) 55-59.

130. M. Yanagita, H. Hoshino, K. Nakayama, T. Takeuchi, Processing of mutated proinsulin with tetrabasic cleavage sites to mature insulin reflects the expression of furin in nonendocrine cell lines, Endocrinology. 133(2) (1993) 639-644.

131. G.D. Simonson, D.J. Groskreutz, C.M. Gorman, M.J. MacDonald, Synthesis and processing of genetically modified human proinsulin by rat myoblast primary cultures, Hum Gene Ther. 7(1) (1996) 71-78.

132. H.C. Lee, S.J. Kim, K.S. Kim, H.C. Shin, J.W. Yoon, Remission in models of type 1 diabetes by gene therapy using a single- chain insulin analogue, Nature. 408(6811) (2000) 483-488.

133. S.-C. Tang, A. Sambanis, Preproinsulin mRNA engineering and its application to the regulation of insulin secretion from human hepatomas, FEBS Letters. 537(2003) 193-197.

134. S. Tanna, M.J. Taylor, G. Adams, Insulin delivery governed by covalently modified lectin-glycogen gels sensitive to glucose, J Pharm Pharmacol. 51(10) (1999) 1093-1098.

135. M.J. Taylor, S. Tanna, P.M. Taylor, G. Adams, The delivery of insulin from aqueous and non-aqueous reservoirs governed by a glucose sensitive gel membrane, J Drug Target. 3(3) (1995) 209-216.

136. S. Tanna, M.J. Taylor, Characterization of model solute and insulin delivery across covalently modified lectin-polysaccharide gels sensitive to glucose, Pharm. Pharmacol. Commun. 4(1997) 117-122.

137. A. Sambanis, Tan, S.A., *Quantitative modeling of limitations caused by diffusion*, in *Methods in Molecular Medicine, Vol. 18: Tissue Engineering Methods and Protocols*, J.R. Morgan, and Yarmush, M.L., Editor. 1999, Humana Press, Inc.: Totowa, NJ.

138. E. Tziampazis, A. Sambanis, Tissue engineering of a bioartificial pancreas: modeling the cell environment and device function, Biotechnol Prog. 11(2) (1995) 115-126.

139. A.M. Shapiro, E.A. Ryan, J.R. Lakey, Pancreatic islet transplantation in the treatment of diabetes mellitus, Best Pract Res Clin Endocrinol Metab. 15(2) (2001)

241-264.

140. A.M. Shapiro, E.A. Ryan, J.R. Lakey, Clinical islet transplant--state of the art, Transplant Proc. 33(7-8) (2001) 3502-3503.

141. A. Kikuchi, T. Okano, Pulsatile drug release control using hydrogels, Adv. Drug Delivery Rev. 54(2002) 53-77.

142. T. Miyata, A. Jikihara, K. Nakamae, A.S. Hoffman, Preparation of poly(2-glucosyloxyethyl methacrylate)-concanavalin A complex hydrogel and its glucose-sensitivity, Macromol. Chem. Phys. 197(1996) 1135-1146.

143. Y. Qiu, K. Park, Environment-sensitive hydrogels for drug delivery, Advanced Drug Delivery Reviews. 53(2001) 321-339.

144. K. Podual, F.J. Doyle, 3rd, N.A. Peppas, Glucose-sensitivity of glucose oxidase-containing cationic copolymer hydrogels having poly(ethylene glycol) grafts, J Control Release. 67(1) (2000) 9-17.

145. K. Podual, F.J. Doyle, 3rd, N.A. Peppas, Dynamic behavior of glucose oxidase-containing microparticles of poly(ethylene glycol)-grafted cationic hydrogels in an environment of changing pH, Biomaterials. 21(14) (2000) 1439-1450.

146. K. Podual, F.J.D. III, N.A. Peppas, Preparation and dynamic response of cationic copolymer hydrogels containing glucose oxidase, Polymer. 41(2000) 3975-3983.

147. C. Malikkides, R. Weiland, On the mechanism of immobilized glucose oxidase deactivation by hydrogen peroxide, Biotech Bioeng. 24(1982) 2419-2439.

148. I. Hisamitsu, K. Kataoka, T. Okano, S. Y., GLucose-responsive gel from phenylborate polymer and poly(vinyl alcohol): Prompt response at physiological pH through the interaction of borate with amino group in the gel, Pharm Res. 14(3) (1997) 289-293.

149. D. Shiino, Y. Murata, K. Kataoka, Y. Koyama, M. Yokoyama, T. Okano, Y. Sakurai, Preparation and characterization of a glucose-responsive insulin-releasing polymer device, Biomaterials. 15(2) (1994) 121-128.

150. D. Shiino, Y. Murata, A. Kubo, Y. Kim, K. Kataoka, Y. Koyama, A. Kikuchi, M. Yokoyama, Y. Sakurai, T. Okano, Amine containing phenylboronic acid gel for glucose-responsive insulin release under physiological pH, J. Control Release. 37(1995) 269-276.

151. J.J. Kim, K. Park, Glucose-binding property of pegylated concanavalin A, Pharm Res. 18(6) (2001) 794-799.

152. J.J.K.a.K. Park, Glucose-binding property of pegylated concanavalin A, Pharm Res. 18(2001) 794-799.
153. E. Hathout, J. Lakey, J. Shapiro, Islet transplant: an option for childhood diabetes? Arch Dis Child. 88(7) (2003) 591-594.

154. L. Falqui, S. Martinenghi, G.M. Severini, P. Corbella, M.V. Taglietti, C. Arcelloni, E. Sarugeri, L.D. Monti, R. Paroni, N. Dozio, G. Pozza, C. Bordignon, Reversal of diabetes in mice by implantation of human fibroblasts genetically engineered to release mature human insulin, Hum Gene Ther. 10(11) (1999) 1753-1762.

155. S.Y. Cheng, J. Gross, A. Sambanis, Hybrid pancreatic tissue substitute consisting of recombinant insulin-secreting cells and glucose-responsive material, Biotechnol Bioeng. 87(7) (2004) 863-873.

156. S.C. Barry, N. Ramesh, D. Lejnieks, W.T. Simonson, L. Kemper, A. Lernmark, W.R. Osborne, Glucose-regulated insulin expression in diabetic rats, Hum Gene Ther. 12(2) (2001) 131-139.

157. J.J. Kim, K. Park, Modulated insulin delivery from glucose-sensitive hydrogel dosage forms, J Control Release. 77(1-2) (2001) 39-47.

158. T.K. Dam, R. Roy, S.K. Das, S. Oscarson, C.F. Brewer, Binding of multivalent carbohydrates to concanavalin A and Dioclea grandiflora lectin. Thermodynamic analysis of the "multivalency effect", J Biol Chem. 275(19) (2000) 14223-14230.

159. M.J. Roberts, M.D. Bentley, J.M. Harris, Chemistry for peptide and protein PEGylation, Adv Drug Deliv Rev. 54(4) (2002) 459-476.

160. S. Zalipsky, Chemistry of polyethylene glycol conjugates with biologically active molecules, Adv Drug Deliv Rev. 16(1995) 157-182.

161. S.J. Stocks, A.J. Jones, C.W. Ramey, D.E. Brooks, A fluorometric assay of the degree of modification of protein primary amines with polyethylene glycol, Anal Biochem. 154(1) (1986) 232-234.

162. O.J. Rolinski, D.J. Birch, L. McCartney, J.C. Pickup, Molecular distribution sensing in a fluorescence resonance energy transfer based affinity assay for glucose, Spectrochim Acta A Mol Biomol Spectrosc. 57(11) (2001) 2245-2254.

163. C.M. Pai, H. Jacobs, Y.H. Bae, S.W. Kim, Synthesis and characterization of soluble concanavalin A oligomer, Biotechnol Bioeng. 41(1993) 957-963.

164. C.M. Pai, Y.H. Bae, E.J. Mack, D.E. Wilson, S.W. Kim, Concanavalin A microspheres for a self-regulating insulin delivery system, J Pharm Sci. 81(6) (1992) 532-536.

165. G.R. Gunther, J.L. Wang, I. Yahara, B.A. Cunningham, G.M. Edelman, Concanavalin A derivatives with altered biological activities, Proc Natl Acad Sci U S A. 70(4) (1973) 1012-1016.

166. D.K. Mandal, C.F. Brewer, Differences in the binding affinities of dimeric concanavalin A (including acetyl and succinyl derivatives) and tetrameric concanavalin A with large oligomannose-type glycopeptides, Biochemistry. 32(19) (1993) 5116-5120.

167. G.H. McKenzie, W.H. Sawyer, The binding properties of dimeric and tetrameric concanavalin A. Binding of ligands to noninteracting macromolecular acceptors, J Biol Chem. 248(2) (1973) 549-556.

168. T. Ueno, K. Ohtawa, Y. Kimoto, K. Sakurai, Y. Kodera, M. Hiroto, A. Matsushima, H. Nishimura, Y. Inada, Polyethylene glycol-modified concanavalin A as an effective agent to stimulate anti-tumor cytotoxicity, Cancer Detect Prev. 24(1) (2000) 100-106.

169. D. Neumann, C.M. Lehr, H.P. Lenhof, O. Kohlbacher, Computational modeling of the sugar-lectin interaction, Adv Drug Deliv Rev. 56(4) (2004) 437-457.

170. The Medtronic MiniMed 2007 Implantable Insulin Pump System. http://www.minimed.com/patientfam/pf_products_implantpump_eu.shtml